ZINC BIOFORTIFICATION OF LENTIL

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Plant Sciences
University of Saskatchewan
Saskatoon

By
Shudhangshu Shekhar Kundu

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis. Requests for permission to copy or to make other uses of materials in this thesis in whole or part should be addressed to:

Head of the Department of Plant Sciences

51, Campus Drive

University of Saskatchewan

Saskatoon, SK S7N 5A8

Canada

ABSTRACT

Lentil (*Lens culinaris* Medik. ssp. culinaris) is an important legume crop in western Canada and a popular nutritious food in the Mediterranean and south Asian regions. Micronutrient deficiency is recognized as hidden hunger in the world. Zinc is essential for all forms of life on the planet and plays a significant role in normal growth, development, and reproduction in plants. Lentil is considered to be one of the cheapest sources of dietary Zn, which plays a major role for normal growth, development, and activity of various enzymes in humans and animals. Lentil production in Saskatchewan contributes to the Canadian economy. Whole and split lentils are exported to many developing countries where Zn deficiency is identified as potential problem. This research was aimed at investigating the potential for biofortification of Zn in lentils. The research objectives were to: i) optimize the amount of lentil seeds required for reliable estimation of Zn concentration, ii) screen representative wild lentil genotypes from all *Lens* species for Zn uptake in lentil seeds during three different seasonal harvests, and iii) study Genotype × Environmental influences on Zn accumulation in intraspecific lentil recombinant inbred lines (RILs) population.

Using flame atomic absorption spectrometry (F-AAS) a wide range of Zn concentration was measured in a collection of lentil genotypes. Seed sample sizes of 0.3 g for wild lentil seeds and 0.5g of cultivated lentil seeds were determined to be the minimum amount required for the precise and repeatable estimation of Zn concentration in lentil seeds.

Field experiments were conducted in 2014 and 2015 for the estimation of genotype and seasonal harvest interactions for seed Zn concentration in *Lens* species in three environments at Saskatoon, Canada. Mature seeds were harvested three times from all individual genotypes during the lentil growing season. All *Lens* species were significantly different for seed Zn concentration. Nonetheless, seed Zn concentration of seasonal harvests was not significantly different in *Lens* species. Limited effects of genotype by harvest interaction were observed for seed Zn concentration in 2014. However, the same trend was not observed in 2015 trial.

Lentil intraspecific recombinant inbred lines (RIL) designated as LR-08 population was made from the cross of CDC Redberry and ILL 7502 and 120 individuals of this population along with parents were evaluated in four environments at Saskatoon during the lentil growing season in 2014-15. Seeds of these individual RIL were analyzed for estimation of Zn

concentration. A wide range of seed Zn concentration (31- 45 mg kg $^{-1}$) variation was observed in RILs of LR-08. The genotype by environmental interaction effects was not significant for seed Zn concentration. However, Genotype by environmental interaction showed significant effects only for thousand seed weight. Thousand seed weight was observed as the most highly stable and heritable trait among all observed traits in LR-08 RIL population. Correlation analysis indicated significant positive correlation between plant height and Zn concentration of lentil seeds in 2015. However, seed Zn concentration was negatively correlated with the days to flowering, days to maturity, and thousand seed weight. Days to flowering and days to maturity were highly correlated.

ACKNOWLEDGEMENTS

Scientific research studies cannot be accomplished by a single individual student but it needs continuous inspiration and sincere guidance from scientific intellectuals. I would like to express my eternal gratitude to my respected supervisor Prof. Albert Vandenberg for his keen interest, guidance, and encouraging motivation during the whole course of my study and in particularly at the times of research and preparation of this thesis. The successful completion of this thesis is also indebted to the valuable guidance of my respected committee members, Drs. Kirstin E. Bett, Jeff Schoenau, and Yuguang Bai. I extend my sincere appreciation to the external examiner Dr. Chris Ambrose for his time and advice for improving the manuscript.

I would like to express my immense gratitude to Dr. Ashutosh Sarker from ICARDA, India for encouraging me in lentil breeding and for introducing me to Dr. Vandenberg for pursuing my study on lentil biofortification program. I would like to appreciate the technical assistance from the members of Crop Science Field Laboratory: Brent Barlow, Thiago Do Prado, and Stacey Wagenhoffer for setting up field trials, Devine De Silva and Alison Sackville for providing seed materials, Barry Goetz for micronutrients analysis, and finally Hamid Khazaei for association mapping study. I am also grateful to Rajib Podder, Li-An Chen, Drs. Shyamali Saha, and Linda Gorim. My thanks also extended to all the faculty, staff, and fellow graduates of the Department of Plant Sciences for their friendship and good wishes for me all the time.

I would like to express my heartfelt thanks to the Natural Science and Engineering Research Council (NSERC) of Canada, Industrial Research Chair Program, Saskatchewan Pulse Growers, and the University of Saskatchewan for financial support to complete this research program.

In the end, I would like to extend my sincere gratitude to my parents for their love, inspiration, and continuous guidance. I wish health and wellness for them!

DEDICATION

This thesis is dedicated to my parents for their patience, support, and sacrifice for my academic career. In addition, I would like to dedicate this research studies to all my well-wishers.

TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGEMENTS iv

DEDICATION v

TABLE OF CONTENTS vi

LIST OF TABLES x

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xv

1. INTRODUCTION AND RESEARCH HYPOTHESES 1

1.1 Introduction 1

1.2 Research Hypotheses 2

2. LITERATURE REVIEW 3

2.1 Lentil 3

2.2 Lentil Seed Size 4

2.3 Lentil Production in Canada 4

2.4 Zinc in Soils 5

2.5 Zinc Uptake and Movement in Plants 5

2.6 Zinc for Plant Nutrition 6

2.7 Zinc in Seeds 7

2.8 Zinc for Human Nutrition 7

2.9 Molecular Marker - QTL Association 8

2.10 Single Nucleotide Polymorphic (SNP) Markers for Lentil 8

2.11 Association Mapping for Crop Improvement 9

2.12 Lentil Biofortification Studies 10

2.13 Justification of Research 11

3. OPTIMIZATION OF SEED ZINC ANALYSIS OF LENTIL SPECIES BY F-AAS 12

3.1 Abstract 12

3.2 Introduction **12**
3.3 Materials and Methods **14**
3.3.1 Apparatus **14**
3.3.2 Reagents and Solutions **14**
3.3.3 Sampling of Seeds **15**
3.4 Procedure **15**
3.5 Statistical Analysis **16**
3.6 Results and Discussion **17**
3.6.1 Method Validation **17**
3.6.2 Seed Amount Optimization for Zn Analysis in Lentil Seeds **20**
3.7 Conclusions **24**
3.8 Acknowledgements **25**
4. SEED ZINC CONCENTRATION AT THREE GROWTH STAGES IN THREE ENVIRONMENTS FOR SEVEN LENTIL SPECIES **26**
4.1 Introduction **26**
4.2 Hypotheses **26**
4.3 Objectives **27**
4.4 Materials and Methods **27**
4.4.1 Plant Material **27**
4.4.2 Phenotyping and Seed Zn Concentration Estimation **27**
4.4.3 Statistical Analysis **28**
4.4.4 Site Description and Environmental Conditions **28**
4.5 Results and Discussion **29**
5. GENOTYPE × ENVIRONMENT INTERACTION ON ZN ACCUMULATION IN SEEDS OF LENTIL INTRASPECIFIC RECOMBINANT INBRED POPULATION **33**
5.1 Introduction **33**
5.2 Hypothesis **33**
5.3 Objective **33**
5.4 Materials and Methods **34**
5.4.1 Plant Material **34**

5.4.2 Phenotyping 34
5.5 Statistical Analysis 34
5.5.1 Phenotypic Data Analysis 34
5.6 Results and Discussion 36
5.6.1 Phenotypic Variation 36
5.6.2 Estimation of Variance Components and Heritability 43
5.6.3 Correlations in 2015 43
5.6.4 Selection of RILs from the Intraspecific RIL populations 44
6. GENERAL DISCUSSION AND CONCLUSIONS 47
6.1 General Discussion 47
6.2 Conclusions 50
6.3 Future Research 51
7. REFERENCES 52
APPENDICES 64
APPENDIX A: ASSOCIATION MAPPING OF ZINC CONCENTRATION IN LENTIL 64
A.1 Abstract 64
A.2 Introduction 64
A.3 Materials and Methods 65
A.3.1 Plant Material 65
A.3.2 Phenotyping 65
A.3.3 Genotyping 65
A.3.4 Statistical Analysis 66
A.3.5 Population Structure and Kinship Calculations 66
A.3.6 Association Analysis 67
A.4 Results and Discussion 67
A.4.1 Phenotypic Data 67
A.4.2 Estimation of Variance Components and Heritability 70
A.4.3 Association Analysis 73

APPENDIX B: GENOTYPE × ENVIRONMENT INTERACTION ON ZN ACCUMULATION IN SEEDS OF LENTIL INTERSPECIFIC RECOMBINANT INBRED POPULATION (LR-26) 77
APPENDIX C: GENOTYPE × ENVIRONMENT INTERACTION EFFECTS ON ZN ACCUMULATION IN SEEDS OF LENTIL INTERSPECIFIC RECOMBINANT INBRED POPULATION (LR-59) 82
APPENDIX D: SEED ZN CONCENTRATIO AT THREE HARVESTS OF 58 WILD LENTIL GENOTYPES IN 2014 87
APPENDIX E: SEED ZN CONCENTRATION AND NODULATION DATA FROM A SUB-SET OF LR-26 AT SASKATOON IN 2014 89
APPENDIX F: SEED ZN CONCENTRATION AND NODULATION DATA FROM A SUB-SET OF LR-59 AT SASKATOON IN 2014 90

LIST OF TABLES

Table 3.1. Wild and cultivated lentil genotypes used for optimizing the estimation of Zn concentration in lentil seeds by F-AAS. 15

Table 3.2. Instrument settings for estimation of Zn concentration by F-AAS. 16

Table 3.3. Accuracy of the method evaluated by comparing the Zn concentration (mg kg^{-1}) of three standard reference materials (certified values) to the average values obtained using the method developed in this study. 18

Table 3.4. Reproducibility of the determination of Zn concentration 20

Table 3.5. Hundred seed weight, mean number of seeds, and mean seed Zn concentration in genotypes of six wild lentil species and cultivated lentil market classes. 21

Table 3.6. Mean comparison of seed Zn concentration (mg kg^{-1}) in four lentil seed sample sizes (0.1, 0.3, 0.5, and 0.7 g) of wild and cultivated lentil genotypes. 22

Table 4.1. Lentil species and genotypes used for measurement 27

Table 4.2. Soil analysis from two field locations at Saskatoon (Crop science field laboratory (CSFL) and Sutherland (STH) farm) in 2014. 29

Table 4.3. Monthly mean air temperature and total precipitation during the lentil growing season (May-August) in 2014-15 29

Table 4.4. Analysis of variance with F-values and significance levels 30

Table 5.1. Soil analysis from three field locations at Saskatoon (Crop science field laboratory (CSFL), Sutherland farm (STH), and Saskatchewan pulse growers farm (SPG)) in 2014. 35

Table 5.2. Simple statistics of LR-08 population for five traits measured at two 36

Table 5.3. Summary of analysis of variance for plant height, days to maturity, thousand seed weight, and zinc concentration for 120 lentil recombinant inbred lines of LR-08 derived from a

cross between ILL 7502/CDC Redberry evaluated at Saskatchewan Pulse Growers (SPG), Sutherland (STH) farms, and Crop Science Field Laboratory (CSFL) in 2014 and 2015..... 42

Table 5.4. Estimates of variance components and broad-sense heritability of plant height, days to maturity, thousand seed weight, and zinc concentration in 120 lentil recombinant inbred line population (LR-08) derived from a cross between ILL 7502/CDC Redberry evaluated at Saskatchewan Pulse Growers and Sutherland farms in 2014 and the Crop Science Field Laboratory and Sutherland farm in 2015. 43

Table 5.5. Pearson correlation coefficients for plant height, days to flowering, days to Maturity, 1000 seed weight, and seed Zn concentration for 120 RILs of LR-08 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015. 44

Table 5.6. Comparison of 10 selected RILs with the highest and lowest seed mean Zn concentration at two locations. 45

LIST OF FIGURES

Figure 3.1. Calibration straight line for standard solutions containing 0, 0.2, 0.4, and 1.2 mg l^{-1} of Zn. 17

Figure 3.2. Comparisons of Zn concentration measured in four sample sizes of the lab check and two standard reference materials. Comparisons were made for each standard reference material separately for Zn. Letters above bars indicate significant differences at $p \leq 0.05$ among different sample weights for each standard. 19

Figure 3.3. Zn concentration in different sample sizes of six wild lentil species. Comparisons were made for each Lens species separately. Different letters above bars indicates significant differences at $p \leq 0.05$ among different sample weights for each species. 22

Figure 3.4. Zn concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.3 g of six wild lentil species. Different letters above bars indicate significant differences at $p \leq 0.05$ among different wild lentil species. 23

Figure 3.5. Zn concentration in different sample sizes of six different market classes of cultivated lentil. Comparisons were made for each market class separately. Different letters above bars indicate significant differences in Zn concentration at $p \leq 0.05$ among different sample weights of cultivated lentil market classes. 24

Figure 3.6. Zn concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.5 g of six market classes of cultivated lentil. Different letters above bars indicate significant differences in Zn concentration at $p \leq 0.05$ among different cultivated lentil market classes. 24

Figure 4.1. Seasonal seed harvests during the lentil growing season. 28

Figure 4.2. Mean zinc concentration (mg kg^{-1}) of lentil seeds harvested at three growing stages in three environments (2014-15). X and Y- axis represent the Zn concentration in seeds and genotypes from seven lentil species. Environments were Crop Science field laboratory (CSFL) - 2014 (a, b, and c), CSFL-2015 (d, e, and f), and Sutherland agriculture farm (STH) -2015 (g, h, and i). Letters indicate the mean Zn concentration in first, second, and third harvest, respectively. Error bars are standard errors of means. 31

Figure 5.1. Frequency distribution of plant height (cm) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 33.3 cm; Sutherland (STH) farm: Mean of RILs = 35.6. 37

Figure 5.2. Frequency distribution of days to flowering of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 47.3; Sutherland (STH) farm: Mean of RILs = 43.4. 37

Figure 5.3. Frequency distribution of days to maturity of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 89.7; Sutherland Agricultural (STH) farm: Mean of RILs = 85.4. 38

Figure 5.4. Frequency distribution of thousand seed weight (g) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2014. Saskatchewan Pulse Growers (SPG): Mean of RILs = 34.0; Sutherland (STH) farm: Mean of RILs = 38.2. 38

Figure 5.5. Frequency distribution of thousand seed weight (g) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 35.3; Sutherland (STH) farm: Mean of RILs = 31.4. 39

Figure 5.6. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2014. Saskatchewan Pulse Growers (SPG): Mean of RILs = 33.6; Sutherland (STH) farm: Mean of RILs = 38.5. 39

Figure 5.7. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 35.9; Sutherland (STH) farm: Mean of RILs = 38.5 40

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CDC	Crop Development Centre
cM	CentiMorgan
DAS	Days after seeding
DTF	Days to flowering
DTM	Days to maturity
DTPA	Diethylene tri-amine penta-acetic acid
F-AAS	Flame atomic absorption spectrometry
LSD	Least significant difference
NIST	National Institute of Standards and Technology
NS	Not significant
PH	Plant height
QTL	Quantitative trait loci
RCBD	Randomized complete block design
RIL	Recombinant inbred line
SAS	Statistical analysis software
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
SNP	Single nucleotide polymorphism
SPG	Saskatchewan Pulse Growers
TSW	Thousand seed weight
[Zn]	Zinc concentration

1. INTRODUCTION AND RESEARCH HYPOTHESES

1.1 Introduction

Everyone on this planet needs zinc (Zn). Zn deficiency is one of the most widespread micronutrient deficiencies in the world, affecting over 30% of total world population (Alloway, 2009; White & Broadley, 2009). Around 2 billion people in the world are affected by Zn deficiency; mostly in developing countries where it is the fifth leading cause of disease and death. Zn deficiency receives global attention, especially in infants and school going children under five years of age. According to the World Health Organization (WHO), about 800,000 people die annually due to Zn deficiency, of which 450,000 are children under the five years of age, representing 4.4% of total annual worldwide deaths of children. Around 60-70% of the population in Asia and Sub-Saharan Africa could be at risk of low Zn intake (Prasad, 2006). Food supplementation, fortification, and biofortification are the three most feasible approaches to address Zn deficiency in humans. The first two approaches require health care systems, infrastructure, and uninterrupted funding; all of these are constraints in most developing countries.

Biofortification of crops with Zn may a sustainable option for alleviating zinc deficiency globally. Application of fertilizers (agronomic biofortification) is the fastest route to alleviate Zn level in diets. However, the most commonly used inorganic Zn fertilizers rapidly become ineffective as it react with soil minerals and organic matter. Genetic biofortification through plant breeding is likely to be the most cost-effective and beneficial route in the long run.

In the last 50 years, the most substantial growth in agricultural production has taken place in cereal crops. Lentil is an ancient pulse crop that has been incorporated into diets across the world. Among all grain legume crops, lentil (*Lens culinaris* Medik.) is recognized as a relatively affordable whole food source with a high content and quality of protein, and micronutrient for human and animal diets compared to other staple foods (Thavarajah et al., 2011). Lentil is a potential candidate crop for Zn biofortification to alleviate Zn deficiency in both developed and developing countries. Genetic investigation is very important for Zn biofortification and bioavailability in lentil. Developing and releasing lentil cultivars with improved micronutrient profiles along with high yield and disease resistance is one of the most important objectives of lentil breeding program at the Crop Development Centre (CDC) of the University of

Saskatchewan, Canada. At this stage, evaluation through phenotype is the main selection process for lentil improvement. Only a few lentil breeding programs are actively involved in the genetic improvement of lentil using wild species, which may have potential for improvement of the nutritional profile as well as disease resistance in lentil breeding. Molecular markers linked to genes that influence increased micronutrient concentration could be the fastest selection procedure to speed up the lentil biofortification programs. Lentil recombinant inbred lines (RILs) have been developed to study the segregation pattern of Zn uptake in the seed. In this research program, a series of studies were conducted to achieve improved bioavailable Zn concentration in lentils.

1.2 Research Hypotheses

i. A minimum amount (number and weight) of lentil seeds is required for accurate and repeatable estimation of Zn concentration by F-AAS.
ii. Zn concentration in seeds varies at different growth stages of lentil plant.
iii. Genotype and environmental interactions influence on Zn accumulation in lentil RIL population.

2. LITERATURE REVIEW

2.1 Lentil

Lentil is one of the oldest pulse crops, belonging to the family Leguminosae (Fabaceae). The cultivated species of lentil is *Lens culinaris* Medik. ssp. *culinaris*. It is used as a pulse (dhal) and grown in rotation with cereals, in both tropical and temperate regions of the globe. Lentils are well accepted as a quality protein source for improving the nutritional quality of cereal-based diets in developing and underdeveloped countries. High nutritional value and short cooking time have increased the importance of lentil production in over 70 countries and lentils are consumed in more than 120 countries.

Lentil is a self-pollinating herbaceous diploid (2n=14) with a haploid genome size of 4,063 Mbp (Arumuganathan & Earle, 1991). Lentil originates from southwest Asia. Lentils are grown in the entire Mediterranean region, central west and south Asia, Ethiopia, Australia, and temperate regions of North and South America. Lentil is a well-adapted cool season legume crop with an indeterminate growth habit. *Lens* species have a longer taproot with number of lateral roots (Sarker et al., 2005). In Canada, lentil is mostly grown in the Brown and Dark Brown soil zone of the prairies and Saskatchewan is the most important lentil producing region.

Lentils flower after a juvenile period of vegetative growth that varies among and within species. Due to having indeterminate growth, maturity (90-110 days), and flowering time varies based on genotype and environmental factors. Lentil plants grow to a height of 15-55 cm. Genetic and morphological variation among lentil genotypes is observed for seed germination, flowering, maturation, height, flower color, stem color, leaves, pubescence, pod size and color, seed size, seed coat color, and cotyledon color among lentil genotypes (Saskatchewan Pulse Growers, 2000).

The taxonomy of *Lens* has undergone numerous changes through several taxonomic revisions base on morphological, cytogenetic, and more recently molecular studies. Based on isozyme studies, (Ferguson et al., 2000) grouped seven taxa into four species: *Lens culinaris* (Medikus) ssp. *culinaris*, *Lens culinaris* ssp. *orientalis* (Boiss.); Ponert *Lens culinaris* ssp. *tomentosus* (Ladiz.) and *Lens culinaris* ssp. *odemensis* (Ladiz.). The secondary gene pool included *Lens ervoides* (Brign.) Grande, the tertiary gene pool included *Lens lamottei* (Czefr.), and *Lens nigricans* (M. Bieb.) Godr. was placed in the quaternary gene pool. Most recently,

Wong et al. (2015) used genotyping by sequencing to place lentil species into four different gene pools. *Lens culinaris*, *Lens tomentosus*, and *Lens orientalis* were considered the primary gene pool, *Lens lamottei*, and *Lens odemensis* were the secondary gene pool, and *Lens ervoides* and *Lens nigricans*, respectively, were considered the tertiary and quaternary gene pools. Lentil is considered as a whole food and Canadian lentils are reported to have Zn concentration of 44-54 mg kg^{-1} and a relatively low concentration of phytic acid (2.5-4.4 mg g^{-1}) a micronutrient bioavailability inhibitor (Thavarajah et al., 2011).

2.2 Lentil Seed Size

Lentil seed size and color are important characteristics in determining the market class. The seed thickness, diameter, and weight are also considered important parameters for optimum seed quality. Lentil seed size has historically been determined by measuring 100 or 1000 seed weight (Erskine et al., 1985; Tullu et al., 2001). Lentil seed size varies across the species of *Lens*, with significant size and weight difference among genotypes from the different center of origins. Abbo et al. (1991) reported large seed weight variation in cultivated lentil, and also observed seed diameter ranging from 3-9 mm. A 15-20 folds seed weight differences has been observed across the group of *Lens* species. Canadian lentil cultivars generally have larger seeds compared to Indian cultivars and wild lentil progenitors. Barulina, (1930) classified cultivated lentils into two sub-species, microsperma (small seeded) and macrosperma (large seeded) which were considered two different lentil biotypes. The seeds of wild lentil species are small compared to cultivated lentil varieties (CFIA, 2016). Ferguson & Robertson, (1999) studied the morphological and phenological variation of 310 accessions of wild *Lens* taxa from the ICARDA germplasm collection. They reported that for cultivated lentil, 100-seed weight (HSW) ranges from 1.6-10.1 g and the wild lentil accessions had much lower HSW than the *Lens culinaris* laboratory standard.

2.3 Lentil Production in Canada

World production of lentil was 4.88 Mt in 2014 and Canada is the largest producer and exporter of lentil (FAOSTAT, 2015). The majority of world lentil production and trade is red cotyledon lentil. Canadian lentil export had a value of CDN$ 2.5 billion in 2015 with an increase of more than 50% over 2013 (Saskatchewan Agriculture Exports, 2015). Besides direct economic benefits, lentil crops can supply significant amount of nitrogen to the soil resource by

fixing nitrogen. Lentil production is most successful when grown in rotation with wheat and other cereal grains which can benefit from higher protein and yield. Gan et al. (2015) reported the cereal-pulse crop rotation increased protein yield by nearly 60% and total grain production increased by more than 35%, and enhanced fertilizer-nitrogen use efficiency by 33% compared to the cereal-summer fallow system.

2.4 Zinc in Soils

Sufficient Zn concentration in soils is very important for good crop production. Severe Zn deficiency in soils can cause severe reduction in crop yield. Zn has emerged as the most widespread micronutrient deficiency in soils. Around half of the cultivated soils in the world are Zn deficient. FAO estimates that 50% of soils growing cereal crops are Zn deficient. Intensive cereal cultivation further decreases soils and grain Zn concentration. Soil conditions that generally caused Zn deficiency in crops include high calcium carbonate, high magnesium and/ or bicarbonate concentrations, high salt concentration, high metal oxides, alkaline pH, low level of organic matter, and soil moisture and prolonged waterlogging. Diethylene triamine pentaacetic acid (DTPA) extractable Zn is used as an indicator of the severity of Zn deficiency in soils. Among the soils factors affecting the solubility of total Zn in soil, pH plays a key role. Soil pH higher than six reduces the available Zn for crops and highly alkaline soils reduce availability of Zn in soil. Soil liming lower the Zn availability compared to acidic conditions and can increase Zn deficiency in crops (Hafeez et al., 2013).

Plants can also suffer from reduced Zn accumulation because of adverse climatic condition such as drought and compaction (Alloway, 2008). Soil temperature also has a significant effect on the rate of Zn mineralization (Takkar & Walker, 1993). Bauer and Lindsay, (1965) reported that Zn deficiency in plant was associated with cool and wet seasons. Temperatures below 16^{o} C were reported to reduce the uptake of Zn in maize (*Zea mays*).

2.5 Zinc Uptake and Movement in Plants

Plant roots absorb available Zn from the soil solution as a divalent cation (Zn^{2+}) or as a Zn-phytosiderophores complex (Singh et al., 2005). At higher pH, Zn is absorbed as monovalent $ZnOH^{+}$ by the root membrane (Marschner, 2011). Zinc uptake through roots has biphasic kinetics, initial binding with the root cell wall, followed by a slower linear transport across the plasma membrane. Available Zn^{2+} efflux movement starts from the external solution to the root

cell wall via diffusion, followed by Zn transport across the plasma membrane. Zinc may pass through the root to the xylem via cytoplasmic continuum of root cells linked by plasmodesmata. Long distance transport of Zn (from the root to storage organs like seeds) uses both intracellular (symplastic) and extracellular (apoplastic) processes that involve a number of dedicated membrane-bound ion transport proteins. Zinc concentration in *Medicago truncatula* is regulated by Zn transporters that control uptake, efflux, and compartmentalization within the plant (Stephens et al., 2011). Ramegowda et al. (2003) reported that over 100 members of zinc interaction protein (ZIPs) families were identified from several higher plants. ZIP proteins act at the plasma membrane surface to move metals and remobilize them from intercellular compartments into the cytoplasm (Colangelo & Guerinot, 2006). Six Zn-regulated transporters (ZRTs) were identified in *Medicago truncatula* (Lo´pez-Milla´n et al., 2004). Stephens et al. (2011) three transporters from six ZRT proteins were characterized for their metal selectivity and identified to transport Zn in *M. truncatula*.

2.6 Zinc for Plant Nutrition

Zn is an essential micronutrient for growth and development of healthy plant during the entire life cycle. Plants need relatively smaller amounts of Zn compared to other micronutrients. Insufficient Zn results in stunted growth and a range of deficiency syndromes in plants. Seeds with higher Zn concentration had improved vigor compared to low levels of Zn (Alloway, 2008). Zn is an important factor for plant growth, stress tolerance, and chlorophyll production (Sharma et al., 2013). Severe Zn deficiency generally shows “little leaf” syndrome (reduction in leaf size) which can results in the “rosette” symptom in dicotyledonous plants and “fan-shaped” stems in monocotyledonous plants. These symptoms can cause 50-70% of the total loss of net photosynthesis (Marschner, 2011), branching suppression (Pandey et al., 2006), and shortened internodes, stunted growth, other symptoms including interveinal chlorosis, and leaf bronzing (Alloway, 2008; Hussain et al., 2011).

Pandey et al. (2006) reported Zn deficiency had significant effects on both lentil seed setting and seed viability, which subsequently had adverse effects on total yield. Zn deficiency can be addressed through the application of Zn fertilizers, natural organic manures, and cultivation of Zn efficient genotypes (Singh, 2008). The sufficiency range of Zn concentration in leaf tissue for most of the crop species is 15-50 ppm, and concentrations below this range level are considered deficiency (Benton, 2002).

2.7 Zinc in Seeds

A high concentration of Zn reserves in seeds is usually an indicator of a healthy plant. Physiological processes controlling micronutrient accumulation in the seed are not yet well understood. Micronutrient accumulation in seeds is a heritable trait and therefore, cultivars could be improved for micronutrient uptake by selective plant breeding. Screening genotypes for genetic variability for seed Zn concentration is the first general step in selective plant breeding. Significant genotypic variation for seed Zn accumulation has been reported in several staple crops: rice (*Oryza sativa*), wheat (*Triticum* spp.), barley (*Hordeum vulgare* L.), maize (*Zea mays*), and common bean (*Phaseolus vulgaris*) (Genc et al., 2002; Hacisalihoglu et al., 2004; Mantovi et al., 2003).

Development of genotypes with high Zn concentration could be a practical solution for increasing yield in Zn deficient soils, especially where farmers are not aware of Zn deficiency and Zn fertilization is not practical (Çakmak et al., 2004). Micronutrient-dense seeds can protect the plant against fungal root diseases, resulting in lower dependence on fungicides (Streeter et al., 2001). Seeds with sufficient micronutrients have the capacity to last until a large root system is developed to compensate in deficient soils.

2.8 Zinc for Human Nutrition

Zn is essential for all aspects of human health. Hotz & Brown, (2004) reported that Zn is critically important for human health and is present in every constituent of the human body. Inadequate intake of one or more micronutrients in the daily diet is referred as ‘hidden hunger’ in human. Zn is the only metal that is required for proper function of all six classes of enzymes (Sharma et al., 2013). More than 300 enzymes with activity in the human body are affected by insufficient Zn supply. Nearly 100 enzymes critically require sufficient Zn for their proper function. Zn is involved in cellular division, synaptic signalling, DNA synthesis, and most of the major metabolic activity in the body (Hotz & Brown, 2004). Inadequate intake and poor absorption of Zn by the human body result in Zn deficiency. Hair loss, memory loss, skin problems, delayed wound healing (Lansdown et al., 2006), and weakness in muscles are the general observed symptoms for Zn deficiency. Zinc deficiency for longer periods may cause weakening of the immune system, infertility, and congenital diseases like acro-dermatitis. Insufficient intake of Zn during pregnancy may cause premature pregnancy (Shah & Sachdev, 2001), increased abortion risk (Jameson et al., 2006), and stunted brain development of the

foetus. In many developing countries, Zn supplementation of normal diets showed significant reduction of diarrhoea and other infectious diseases among school age children (Walker et al., 2009). Monotonous consumption of cereal-based foods in the developing countries has been shown to be a key reason for high prevalence of Zn deficiency. Beef, chicken, pork, peanuts, almonds, walnuts, oats, and dairy products including milk, yogurt, and cheese are rich in Zn content

2.9 Molecular Marker - QTL Association

Molecular markers are considered to be effective tools for molecular assisted plant breeding. A major goal of plant molecular genetics is identification of genomic regions that control the phenotypes (Weigel & Nordborg, 2005). Molecular markers can help breeders to distinguish individual genotypes, thereby improving the efficiency of selection. Individual genotypes that have gone through the natural selection and recombination at greater levels reveal higher mapping resolution of Quantitative trait loci (QTL) for the particular trait. High-resolution mapping would allow identification of the genes responsible for target traits. Most traits of evolutionary importance in agriculture are controlled by multiple genes. Breeders can use QTLs association to select desired genotypes by using markers with low heritability. QTL mapping on chromosomes using marker-trait association has become a feasible research strategy in crop improvement. In the past two decades, QTL identification based on genetic recombination by linkage analysis has been studied in many crops. An abundant number of QTLs have been identified for important agronomic traits in wheat and field pea (Li et al., 2002; Pozniak et al., 2007; Scoles et al., 2010). These QTLs are generally located within large intervals on different chromosomes. Association mapping can enhance QTL information for marker-assisted selection in plant breeding. QTL mapping facilitates progress in comparative mapping between related species. Marker from one species may be used for identification of new syntenic marker in other related plant species. QTL mapping also helps to identify loci for quantitative and qualitative traits based on segregating populations.

2.10 Single Nucleotide Polymorphic (SNP) Markers for Lentil

Among polymorphic markers, SNPs are the abundant type of marker in any genome of a species. They are being considered as one of the most powerful tool in positional cloning, genetic mapping, diversity analysis, and AM for the trait of interest in many crop species

(Rafalski, 2010). Recent advancement in plant biotechnology has sped up the discovery of the abundant type of SNP in almost all crop species. SNP markers are generally bi-allelic, and the availability of abundant SNP helps to build SNP haplotypes for the crop species. Gene-based SNP marker can be developed from the discovery of SNPs in candidate genes for the traits of interest. Gene-based SNP markers can be used as causative SNPs for the specific traits. In legume crops, gene-based polymorphic markers have been used for developing transcript maps in soybean and chickpea (Choi et al., 2007; Gujaria et al., 2011). Rafalski, (2002) reported that expressed sequence tags (ESTs), shotgun genomic libraries, and PCR amplicons are the most widely used methods for SNP discovery in crop plants (Rafalski, 2002). The sequenome platform has been used in SNP validation studies in highly polyploidy species like sugarcane (Bundock et al., 2009), association with common bacterial disease in common bean (Shi et al., 2011), marker assisted selection in soybean breeding (Shi et al., 2011), and assessing genetic diversity in castor bean (Foster et al., 2010). SNPs have been used for developing genetic maps for crop improvement in various crop species (Gupta et al., 2008). SNP markers are commonly used in association mapping for genotype and phenotype association in crops. Correlation between SNP markers, traits and genes are being statistically accessed in both distantly related and unrelated genotypes of different crop species. Sharpe et al. (2013) reported 1,536 bi-allelic SNP markers discovery from genotypes of *Lens culinaris* by using highly a parallel allele-specific Illumina Golden Gate SNP assay. These 1,536 SNP markers can be screened across the collection of genotypes from lentil diversity panel for the association between marker and trait of interest.

2.11 Association Mapping for Crop Improvement

Association mapping (AM) is a feasible approach for the molecular dissection of complex genetic traits and human diseases (Churchill et al., 2004). Because of rapid progress in population and molecular genetics, AM has been used increasingly over the last decade for the study of complex genetic traits. Association mapping was first used for the dissection of the genetic basis for complex human diseases. Linkage disequilibrium (LD) indicates the non-random association of alleles at different loci within the genome. More recently, AM has been used successfully for identification of genetic markers associated with specific phenotypes in various plant species. It involves assessment of individuals in a population structure for trait-marker association. Both linkage mapping and AM identify polymorphisms that associate with

functional alleles through. Association mapping analyses typically include a set of diverse individuals that encompass the genetic variation of multiple historical recombination events of the crop species. Multiple historical levels of recombination accumulated in landraces, natural populations, breeding material and commercial varieties increase the QTL resolution. This ability to determine a marker-trait association has given the AM a unique preference over linkage mapping. Significant associations have been identified between RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeat), and SNP (single nucleotide polymorphism) markers with various morphological traits in maize, rice, barley, wheat, soybean, and common bean (Agrama et al., 2007; Galeano et al., 2012; Jun et al., 2008; Pozniak et al., 2007; Remington et al., 2001; Xia et al., 2013). These studies reported AM to be an effective tool for QTL research. Association mapping based on the linkage disequilibrium is adapted for multiple alleles with higher resolution mapping. It targets multiple alleles at individual loci for a large number of traits. Rare alleles (allele frequency < 5%) in the population structure are treated as missing data which controls the rate of false association in AM analysis (Breseghello & Sorrells, 2006). The mixed linear model is used in AM for controlling the rate of both false-positives and false-negatives, for example in AM of wheat (Yao et al., 2009). The mixed linear model considers the relative kinship by marker based estimation from individual descents.

Complex breeding population created within the germplasm can be the major obstacle in marker-trait association for many crop species (Flint-Garcia et al., 2003). Unequal distribution of alleles within subpopulation of breeding populations may result in the spurious association between marker and trait (Lander & Schork, 1994). Pritchard et al. (2000) reported the use of a Bayesian model to screen unlinked markers throughout the whole genome for successful detection of the population structure. Epistasis effect and alleles of large effect at other loci may mask the functional variation. The allelic diversity that provides functional variation plays an important role in selection by breeders.

2.12 Lentil Biofortification Studies

Biofortification is the process of increasing bioavailability of essential micronutrients in the edible parts of the plant by traditional plant breeding or genetic engineering (White & Broadley, 2005). Plant breeding is a sustainable and recognized concurrent strategy for alleviating micronutrient malnutrition, along with supplementation, dietary diversification,

fortification, and disease reduction. Crop improvement through plant breeding ensures both plant and human nutrition. Plant breeding holds promise for enhancement of micronutrient profile in crop species in a way that is more economically and environmentally sustainable (Kannenberg & Falk, 1995). Previous research investigations have reported biofortification of many crops such as wheat, rice, maize, cabbage, canola, and common bean.

Exploitation of genetic variation is the main basis of biofortification through plant breeding for improving crop cultivars (Ortiz-Monasterio et al., 2007). Wild species of the genus *Lens* have important potential for genetic improvement of resistance to abiotic and biotic stresses, and quality factors related to seed coat pattern, seed coat color, seed coat color retention, Zn, Fe, and Se uptake (Tullu et al., 2013). Crop wild relatives may have value through breeding to deal with the increasingly negative impacts of human population growth and climatic changes. To identify the individual genes responsible for expression of the target trait, contrasting parents from different environments can be used for breeding populations (Rossi et al., 2009). Phenotypic variations are likely to be significantly associated with the polymorphic trait in large germplasm collections. Identification of single nucleotide polymorphism (SNP) markers facilitated by genome wide association mapping can lead to the discovery of genes controlling the trait. A large number of loci significantly associated with phenotypic variation in Zn, copper, arsenic, and molybdenum were identified in rice (Norton et al., 2014). The seven wild species of *Lens* show wide variability in both phenotype and genotype. ICARDA maintains a core collection of 577 wild lentil accessions (Sarker et al., 2001, 2005). The CDC at University of Saskatchewan has received about 400 accessions of the ICARDA collection.

2.13 Justification of Research

Malnutrition due to micronutrient deficiency occurs primarily in developing countries. Food supplementation is a costly approach for overcoming the Zn deficiency in most developing countries. Well-balanced micronutrient composition and relatively low cost leads to almost universal acceptance of lentil in the human diet. Therefore, lentil is a potential staple crop for biofortification since it is already included in daily diets in many developing countries. Genetic strategies of breeding for biofortification in nutrient dense yet inexpensive pulse crops like lentil may contribute to sustainable approaches for dealing with Zn deficiency in humans.

3. OPTIMIZATION OF SEED ZINC ANALYSIS OF LENTIL SPECIES BY F-AAS

The information in this chapter was submitted as a contribution in a manuscript submitted to ***Communications in Soil Science and Plant Analysis*** on March 07, 2016, for publication as "Optimizing zinc and iron analysis of seed samples of lentil species". The submitted manuscript is still in review.

3.1 Abstract

Zinc (Zn) deficiency affect billions of people globally and has a direct impact on human health. Zinc concentration in plant materials are commonly analyzed by flame atomic absorption spectrometry (F-AAS). This research describes the optimal analysis method for precise Zn analysis of lentil seeds from the species of the genus *Lens*. Estimation of Zn concentration in the whole lentil seeds by F-AAS is a destructive procedure as it includes nitric acid digestion of the seeds prior to instrument analysis. The procedure proposed for the determination of Zn concentration uses the minimum amount of lentil seed of all lentil species with suitable standard reference materials. The relative standard deviations of the method were found to be around 5% for both Zn. The minimum amount of 0.3 g of wild and 0.5 g of cultivated lentil seed samples were identified as needed for accurate and precise estimation of Zn concentration.

3.2 Introduction

Flame atomic absorption spectrometry (F-AAS), inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) are the three most commonly used analytical techniques for the determination of trace metal elements in plant tissues. F-AAS is the most used instrument for micronutrient analysis in clean and complex matrix samples (Abarca et al., 2001) because of its lower cost, easier accessibility, and analytical performance. Compared to other time-consuming and laborious digestion procedures, HNO_3-H_2O_2 digestion procedure is the most frequently used digestion procedure for trace elements analysis.

According to the Statistical Bureau of the Food and Agriculture Organization (FAO) of the United Nations, lentil is the fourth most important pulse crop, currently grown in all continents except Antarctica and cultivated in more than 52 countries in the world (FAOSTAT, 2016). Lentil is proven to be a beneficial crop for soil, human, and animal health. Lentil plays an important role in soil fertility owing to its ability to fix atmospheric nitrogen. The nutritional

profile of lentil seeds is recognized as a good food source for human nutrition across the world. Around 60-70% of the population in Asia and Sub-Saharan Africa could be at risk of low Zn intake (Prasad, 2006). Micronutrient deficiencies mainly result from consumption of daily diets with an insufficient concentration of minerals to meet daily dietary requirements. Lentil is a well-known cheap source of quality staple food for poor and vegetarian populations throughout Western Asia, the Middle East, North Africa, and the Indian subcontinent. This nutritional profile clearly indicates its great potential as a nutrient rich food source for developing and developed countries. Cabrera et al. (2003) studied micronutrient concentrations in seven different legumes and found the average Zn concentration in lentil seeds was 56.2 mg kg^{-1}, considerably higher than other legumes such as *Cicer arietinum* (39.2 mg kg^{-1}), *Vicia faba* (41.2 mg kg^{-1}), and *Phaseolus vulgaris* (46.9 mg kg^{-1}).

Wild relatives of crop species have been a valuable source of resistance to abiotic and biotic stresses (Tullu et al., 2010). Wild lentil species are being used for increasing the genetic diversity in cultivated lentil. Genetic resources of wild lentil species originating from different parts of the world revealed higher variation in Zn concentration in seed (Sarker et al., 2007). The accumulation of micronutrients in lentil seeds depends on growing location, genotype, and genotype by location effects. Karaköy et al. (2012) found Zn concentration in Turkish lentil landraces ranges from 42-73 mg kg^{-1}. Zn concentration in Canadian lentil cultivars ranges from 44-54 mg kg^{-1}. Thavarajah et al. (2011) reported a wide range of variation in total Zn concentration ranging from 22-77 mg kg^{-1} in 1200 lentil genotypes including breeding lines, wild, and lentil land races of red and green lentils. Wild lentil seeds and seeds of their interspecific hybrids are often difficult to produce, however, are available only in small quantities from seed resources, making it difficult to assess micronutrient levels.

This study was initiated to assess the concentration of Zn in lentil genotypes available at the Crop Development Centre, University of Saskatchewan, Canada. Micronutrient concentration measurement is destructive; therefore the specific goals of this experiment were: (1) to determine the minimum amount of seeds required for precise estimation of Zn in lentil seeds by F-AAS and (2) to validate a quick and simple analytical method for the estimation of Zn concentration in whole lentil seeds. To our knowledge, this experiment is the first to identify the minimum amount of wild and cultivated lentil seeds necessary to analyze the accurate concentration of Zn in lentil seeds by using the most accessible and cheap analytic technique F-AAS.

3.3 Materials and Methods

3.3.1 Apparatus

An electronic seed counter (ESC-1, Agriculex Inc., Guelph, Canada) was used to count lentil seed samples. The seed weight of lentil genotypes was determined by counting 100 seeds (at 12% moisture content) with convertible electronic balance. Estimations of all metal ion concentrations were performed using an Analytikjena (Jena, Germany), novAA®300 flame atomic absorption spectrometer (AAS) equipped with a computer processor. Deuterium background correction was used with Zn hollow-cathode lamps as radiation sources. Operating conditions recommended by the manufacturer were used throughout the experiment. To maximise the absorbance signal for each metal burner, height, and acetylene-air flow rate were adjusted by aspirating the analyte solution. To maintain discrete volume sampling, a final volume of 100 µl of analyte solution was injected automatically into the flame of the spectrometer through the nebulizer by sample aspiration tubing. Absorbance signals were measured in peak area mode by the spectrophotometer reader. Other instrumental parameters of this spectrophotometer for the estimation of Zn concentration are summarized in Table 3.2.

3.3.2 Reagents and Solutions

All reagents were analytical grade and distilled and deionized water that was further purified by nanopure high purity water (electrical resistivity of 16.0 MΩ cm^{-1}) (Barnstead, Massachusetts, USA). Laboratory glassware was kept in 10% (v/v) HNO3 for overnight and subsequently rinsed four times in distilled water followed by oven drying to avoid contamination. Stock standard solutions of Zn (1000 mg l^{-1}) were obtained from VHG, Manchester, USA. Working standard solutions were prepared by appropriate dilution of the standard stock solutions. A standard solution of Zn was used for calibration. Different concentrations of Zn (0.0, 0.2, 0.4, 1.2, and 1.6 mg l^{-1}) working standard solutions were used to confirm F-AAS accuracy. The standard stock solutions concentration calibration curves were linear (r^2 = 0.9995). Concentrated nitric acid, hydrochloric acid and hydrogen peroxide used in the digestion procedure were supplied by Fisher Chemicals and Anachemia, respectively. Four standard reference materials (tomato leaves (NIST.1573a), durum wheat (NIST.8436a), bovine liver (NIST 1577a), and rice flour (NIST 1568a)) supplied by National Institute of Standards and Technology (NIST, USA) were used as standards to validate the laboratory standard.

3.3.3 Sampling of Seeds

Six wild lentil genotypes (one representing each of the six species of genus *Lens*) and six popular cultivated lentil genotypes from *Lens culinaris* (one representative accession from each of the six most important market classes produced in Canada) were used in this study (Table 3.1). Seeds of wild lentils were grown at Crop Science Field Laboratory, Saskatoon in 2013. The seed samples of six cultivars were collected from the Lentil Regional Varietal Trial, 2013, at Limerick, Saskatchewan.

Table 3.1. Wild and cultivated lentil genotypes used for optimizing the estimation of Zn concentration in lentil seeds by F-AAS.

Wild *Lens* species and genotypes		Cultivated lentil market classes and genotypes	
Species	Genotype	Species	Genotype/market class
Lens orientalis	IG 72611	*Lens culinaris*	CDC Robin/extra small red
Lens tomentosus	IG 72613	*Lens culinaris*	CDC Maxim/small red
Lens lamottei	IG 110813	*Lens culinaris*	CDC KR-1/large red
Lens odemensis	IG 72760	*Lens culinaris*	CDC Viceroy/small green
Lens ervoides	IG 72815	*Lens culinaris*	CDC Greenland/large green
Lens nigricans	IG 116024	*Lens culinaris*	CDC QG-2/green cotyledon

3.4 Procedure

Zinc concentration in the lentil seeds was measured as mg kg^{-1}. Zinc concentration in whole lentil seeds was measured to assess the validity of proposed digestion and analytical methods. Total Zn concentrations in each replicated lentil seed sample was measured by HNO_3-H_2O_2 digestion followed by F-AAS analysis. Whole lentil seed samples were digested using the modified procedure described by Lintschinger et al. (2000).Whole seed samples were thoroughly washed with distilled-deionized water to remove surface contaminants and then air-dried Samples were weighed separately into 0.1, 0.3, 0.5, and 0.7 g which were placed into specific digestion glass tubes (30 ml) of the Vulcan 84 automated digestion chamber. Every analysis set consisted of four blanks and four laboratory standards within a set of 84 digestion tubes. Each digestion tube had 6 ml of concentrated nitric acid (HNO_3) injected into it. The digestion plate temperature was raised to 86°C and then samples were allowed to digest for 45 min. Then 5 ml of hydrogen peroxide (H_2O_2) were injected to each digestion tube and digestion continued for 65

min. At this point 3 ml of 6M HCl was added to all tubes. The tubes were left in the digestion chamber for another 5 min to complete the digestion step. Digested samples were cooled for 45 min, followed by volume adjustment to 25 ml with distilled-deionized water at room temperature (22°C) and then transferred to analysis tubes. Blanks were prepared in the same way but without sample addition. Six ml of digested solution was used each time to determine Zn concentration by F-AAS. Samples, standard working solutions, blanks and standard reference materials were measured by F-AAS under the same instrumental conditions (Table 3.2).

Table 3.2. Instrument settings for estimation of Zn concentration by F-AAS.

Parameter	Zn
Wave length (nm)	213.9
Slit width (nm)	0.5
Light source	Zinc hollow cathode lamp
Power supply (mA)	4
Flame, flow setting (l min^{-1})	Air (6.67), Acetylene (0.75)
Integration time (s)	3
Usable burner height (mm)	4-10

3.5 Statistical Analysis

The experiment was set up in a completely randomized design with four replications. Analysis of variance (ANOVA) was used to determine the concentration of Zn variation in different lentil genotypes using the Mixed model procedure (PROC MIX) of SAS software version 9.4 (SAS institute Inc., Cary, NC, USA). Average concentrations were separated by both genotype and sample size using Fisher's protected LSD procedure and level of significance was declared at $P \leq 0.05$ and 0.01. Contrast statistical analysis was performed using SAS covariance contrast (least squares means) to compare the different lentil seeds sample sizes with one another.

3.6 Results and Discussion

3.6.1 Method Validation

Quality of an analytical method, especially for quantitative analysis is established by its validation. Background knowledge of calibration linearity, accuracy, recovery percentage, precision, and detection limit are the main criteria for assessment of methodology for quantitative analysis of micronutrients.

Four standard solutions of Zn concentration were employed to study the linearity of absorbance response. The calibration curves for different standard solutions were drawn after setting the parameters of F-AAS (Table 3.2) at optimum levels. A linear relationship was obtained for both Zn by plotting each standard solution concentration (0, 0.2, 0.4, and 1.2 mg l^{-1}) against absorbance of Zn (Figure 3.1).

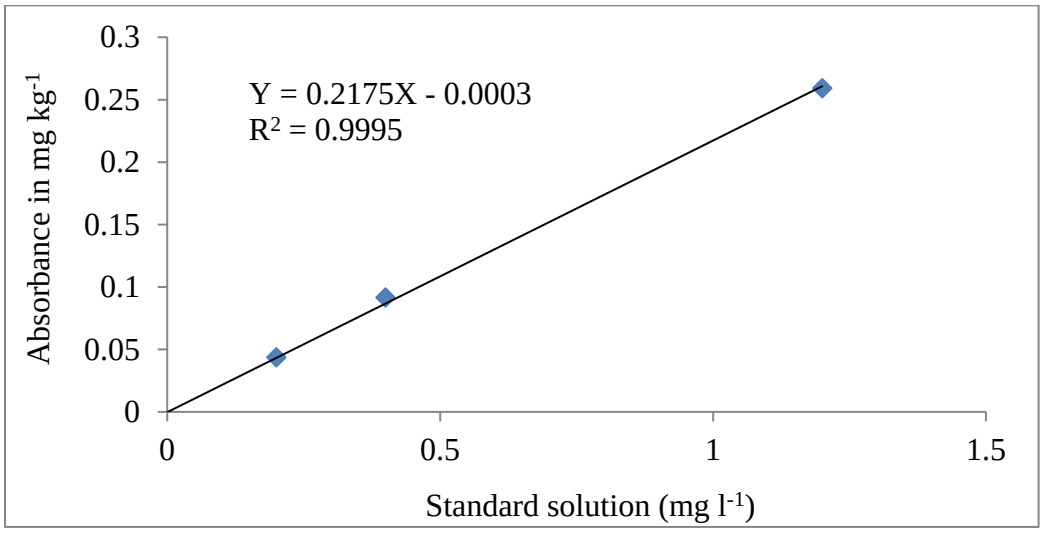

Figure 3.1. Calibration straight line for standard solutions containing 0, 0.2, 0.4, and 1.2 mg l^{-1} of Zn.

The accuracy of the analytical method used in the study was assessed by preparing the same quantity of standard reference materials in a similar matrix followed by digestion and quantification of Zn by F-AAS. Ghaedi et al. (2013) reported relative standard deviation for Zn concentration of about 4% and that recovery above 90% indicates that the analytical method is reliable. Precision and performance of the determination method of Zn concentration in tea samples was considered satisfactory at 7.8 relative standard deviation and 96-98% recovery

(Soylak et al., 2007). In this current study, Mean recovery (% R) of Zn for three standard reference materials with certified values from NIST ranged from 96.3-97.3% and 90.3-101.1%, respectively (Table 3.3).

Table 3.3. Accuracy of the method evaluated by comparing the Zn concentration (mg kg^{-1}) of three standard reference materials (certified values) to the average values obtained using the method developed in this study.

Standard reference material	Certified values (mg Zn kg^{-1})	Average values (mg Zn kg^{-1})	Recovery (%)	Relative standard deviation (%)
Tomato leaves (NIST 1573a)	30.8±0.8	29.6	96.2	4.1
Durum wheat (NIST 8436a)	22.7±1.7	21.9	96.6	5.2
Rice flour (NIST 1568a)	19.4±0.3	18.7	96.4	3.2

Note: Average value of four estimated values of standard reference materials using a similar matrix.

Two standard reference materials, bovine liver (NIST 1577a) and rice flour (NIST 1568a) were used, and yellow lentil was included as a laboratory standard. Four different sample sizes (0.1, 0.3, 0.5, and 0.7 g) of two different standard materials along with the laboratory standard (yellow lentil) were compared under the same instrumental conditions. Among the four sample sizes, 0.1 g showed significant differences with other three sample sizes for Zn concentration (Figure 3.2).

The internal repeatability was measured under the same instrumental settings to evaluate the precision of this analytical method. The same instrumental settings were used to evaluate the precision of this analytical method. Repeatability of this method was assessed by analyzing four different samples (each 0.5 g of CDC Robin) with two replications prepared individually on the consecutive days with the same equipment by the same operator. The relative standard deviation (R.S.D.) of four different samples prepared by the same operator was 0.3%, indicating acceptable repeatability of this method of analysis.

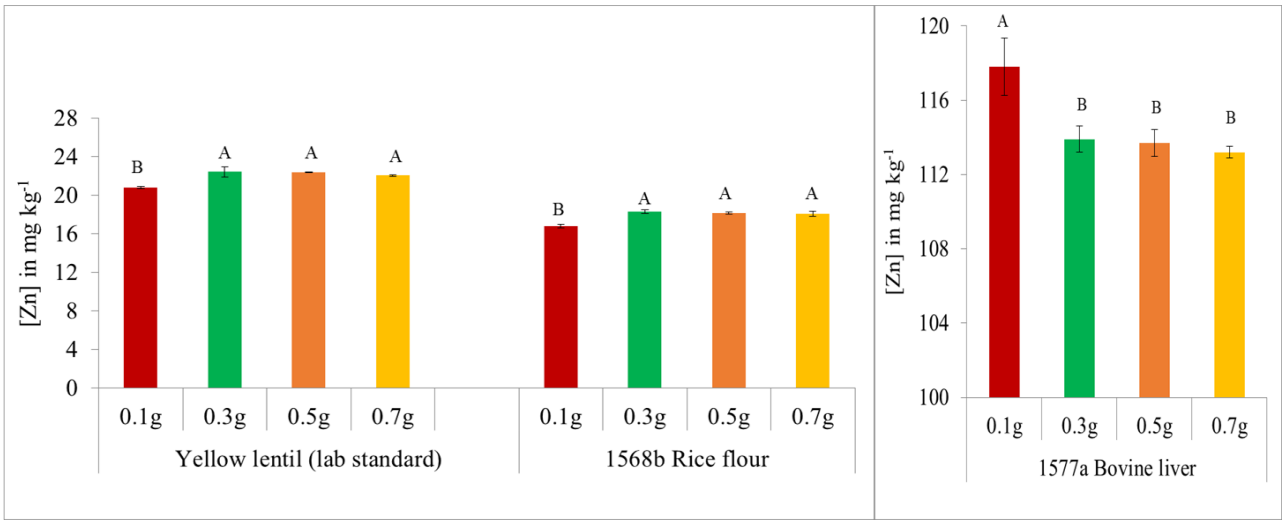

Figure 3.2. Comparisons of Zn concentration measured in four sample sizes of the lab check and two standard reference materials. Comparisons were made for each standard reference material separately for Zn. Letters above bars indicate significant differences at $p \leq 0.05$ among different sample weights for each standard.

The internal reproducibility of the method was estimated by analyzing two different lots of samples prepared over four consecutive days by different operators. Four samples from lot 1 were analyzed on four consecutive days by the same operator (day-to-day fluctuation) under the same instrumental conditions. The relative standard deviation for day-to-day fluctuation was 0.2% (Table 3.4) for seed [Zn]. Four samples from lot 2 were analyzed on two consecutive days by another operator (analyst-to-analyst fluctuation). The relative standard deviation for analyst-to-analyst fluctuation was 0.8% for Zn. Both relative standard deviations for day-to-day and analyst-to-analyst fluctuations showed good reproducibility of this analytical method.

Table 3.4. Reproducibility of the determination of Zn concentration of two lots of samples prepared by two analysts over 4 days.

Lot 1

Analyst	Day	Mean [Zn] (mg kg^{-1})
A	1	33.6
A	2	30.4
A	3	28.9
A	4	34.4
Mean		31.9
R.S.D.* (%)		0.2

Lot 2

Analyst	Day	Mean [Zn] (mg kg^{-1})
A	5	31.9
A	6	31.6
B	7	33.1
B	8	32.9
Mean		32.4
R.S.D*. (%)		0.8

Note. Seed Zn concentration reported here is the mean of two digested solutions run through the F-AAS *R.S.D., Relative Standard Deviation.

3.6.2 Seed Amount Optimization for Zn Analysis in Lentil Seeds

Large differences for Zn concentration were observed both in wild and cultivated lentil genotypes. Weight of 100 seeds of each lentil genotype is reported in Table 3.5. Based on 100 seeds weight, wild lentil species were subdivided into large (>1 g) and small (<1 g) seeded species (Figure 3.4). Zn concentration ranged from 30-41 mg kg^{-1} in wild lentil genotypes. *Lens lamottei* (IG 110813), which belongs to secondary gene pool had the highest Zn concentration and was significantly ($p \leq 0.05$) different from all other wild genotypes (Figure 3.3-4). However, seeds of *Lens odemensis* (IG 72760) of the secondary gene pool had the lowest Zn concentration (Figure 3.4).

Table 3.5. Hundred seed weight, mean number of seeds, and mean seed Zn concentration in genotypes of six wild lentil species and cultivated lentil market classes.

Lens species	Genotype	Hundred seed weight (g)	Mean number of seeds (0.3 g)	Mean [Zn] (mg kg^{-1})
Lens orientalis	IG 72611	1.4	21	36
Lens tomentosus	IG 72613	1.2	26	35
Lens lamottei	IG 110813	1.3	22	40
Lens odemensis	IG 72760	0.7	44	29
Lens ervoides	IG 72815	0.5	61	37
Lens nigricans	IG 116024	0.5	54	38
Lens species	Genotype/market class	Hundred seed weight (g)	Mean number of seeds (0.5 g)	Mean [Zn] (mg kg^{-1})
Lens culinaris	CDC Robin/extra small red	2.9	18	29
Lens culinaris	CDC Maxim/small red	3.9	12	39
Lens culinaris	CDC KR-1/large red	5.4	9	29
Lens culinaris	CDC Viceroy/small green	3.1	15	30
Lens culinaris	CDC Greenland/large green	6.9	7	37
Lens culinaris	CDC QG-2/green cotyledon	3.2	17	28

For wild lentils, statistical analysis among different seed sample sizes showed 0.1 g seed samples were always significantly higher ($p \leq 0.001$) compared to other three larger sample sizes (0.3, 0.5, and 0.7 g) (Table 3.6). Seed Zn concentrations were not significantly different among the larger seed sample sizes (0.3, 0.5 and 0.7 g) (Figure 3.3). Contrast statistical analysis indicates 0.1 g seed sample size was significantly different from other sample sizes for Zn concentration estimation. Therefore, estimation of Zn concentration using the same digestion matrix with 0.3 g of seeds from wild lentil species is deemed more precise and reliable. This would help to reduce seed requirements, analysis time and cost rather than analyzing Zn concentration separately. Sample sizes of 21-26 seeds of the larger seeded wild lentils (*Lens lamottei, Lens tomentosus,* and *Lens orientalis*) and 44-61 seeds of small seeded wild lentil (*Lens nigricans, Lens odemensis,* and *Lens ervoides*) appear sufficient for reliable determination of Zn concentration in wild lentils by F-AAS (Table 3.5).

Table 3.6. Mean comparison of seed Zn concentration (mg kg^{-1}) in four lentil seed sample sizes (0.1, 0.3, 0.5, and 0.7 g) of wild and cultivated lentil genotypes.

Wild lentil species				Cultivated lentil genotypes			
Seed sample size (g)	df	Mean [Zn] (mg kg^{-1})	P ≥ F	Seed sample size (g)	df	Mean [Zn] (mg kg^{-1})	P ≥ F
0.1	92	39	<.0001	0.1	92	41	<.0001
0.3	92	34	<.0001	0.3	92	34	<.0001
0.5	92	34	<.0001	0.5	92	32	<.0001
0.7	92	34	<.0001	0.7	92	30	<.0001

Note. ***df***, degrees of freedom.

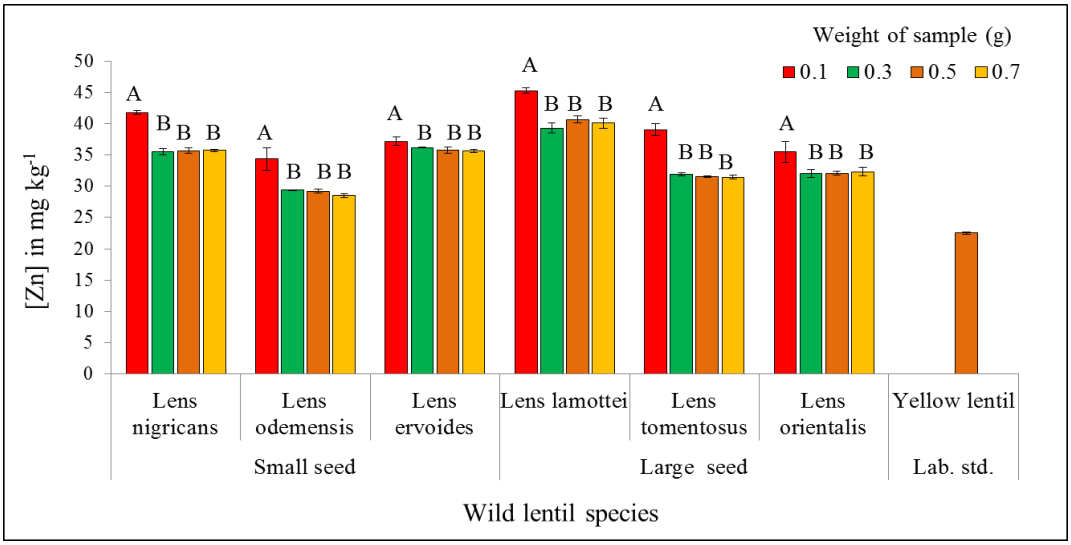

Figure 3.3. Zn concentration in different sample sizes of six wild lentil species. Comparisons were made for each Lens species separately. Different letters above bars indicates significant differences at $p \leq 0.05$ among different sample weights for each species.

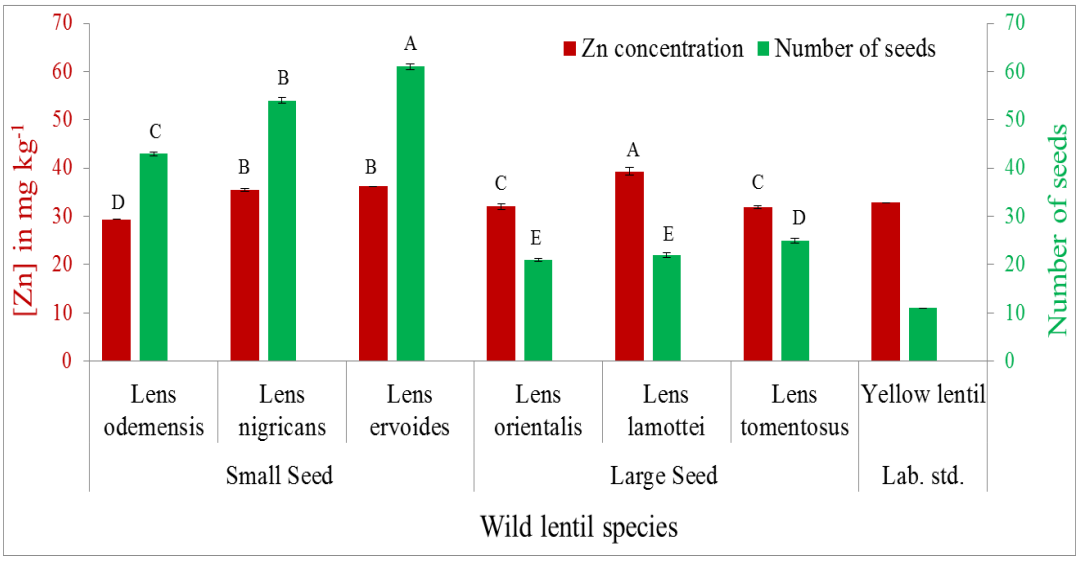

Figure 3.4. Zn concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.3 g of six wild lentil species. Different letters above bars indicate significant differences at $p \leq 0.05$ among different wild lentil species.

Significant Zn concentration differences were also observed in different cultivated lentil genotypes. In the six cultivated lentil genotypes, Zn concentration ranged from 29 to 39 mg kg^{-1}. Small red genotype (CDC Maxim) showed the highest Zn concentration and was significantly different ($p \leq 0.05$) from other cultivated lentil genotypes. In most cases, except extra small red - the smallest seed sample size (0.1 g) of different cultivated lentil genotypes showed significantly higher Zn concentration than all other sample sizes (Table 3.6; Figure 3.5). Both small red and large green had the highest seed Zn concentration. However, green cotyledon genotype (CDC QG-2) had the lowest concentration of Zn (Figure 3.6). Average Zn concentration in different sample sizes of different market classes were shown in Table 3.5. Contrast statistical analysis among different seed sample sizes from cultivated lentil genotypes revealed that 0.5 g of whole lentil seed was more reliable than 0.3 g seed sample size for precise estimation of Zn concentration. This might be due to lower number of seeds in 0.3 g of seed sample sizes which capture lesser seed variability than 0.5 g seed sample size of cultivated lentil genotypes. A seed number of 7-18 seeds (0.5 g) were reliable for precise estimation of Zn concentration in cultivated lentil genotypes (Table 3.5).

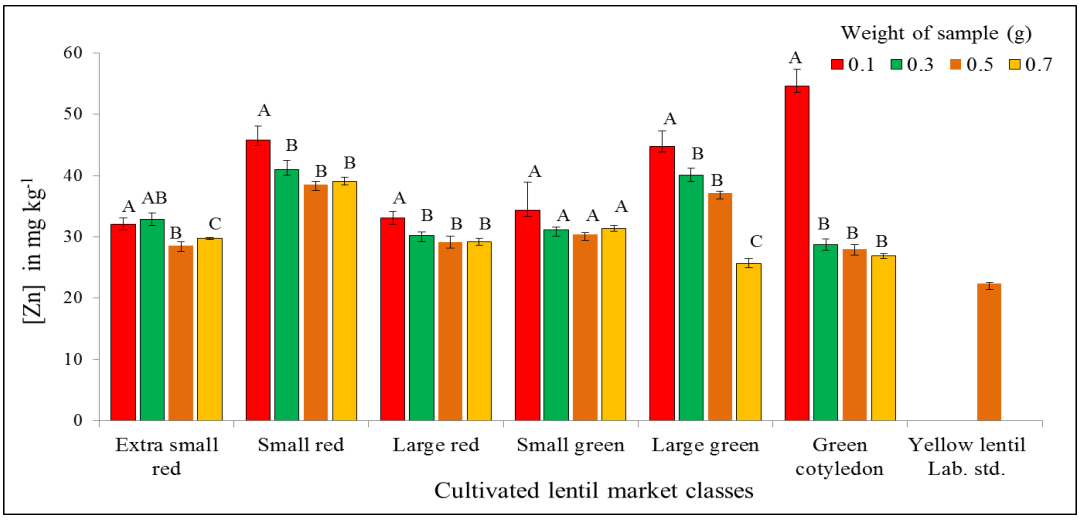

Figure 3.5. Zn concentration in different sample sizes of six different market classes of cultivated lentil. Comparisons were made for each market class separately. Different letters above bars indicate significant differences in Zn concentration at $p \leq 0.05$ among different sample weights of cultivated lentil market classes.

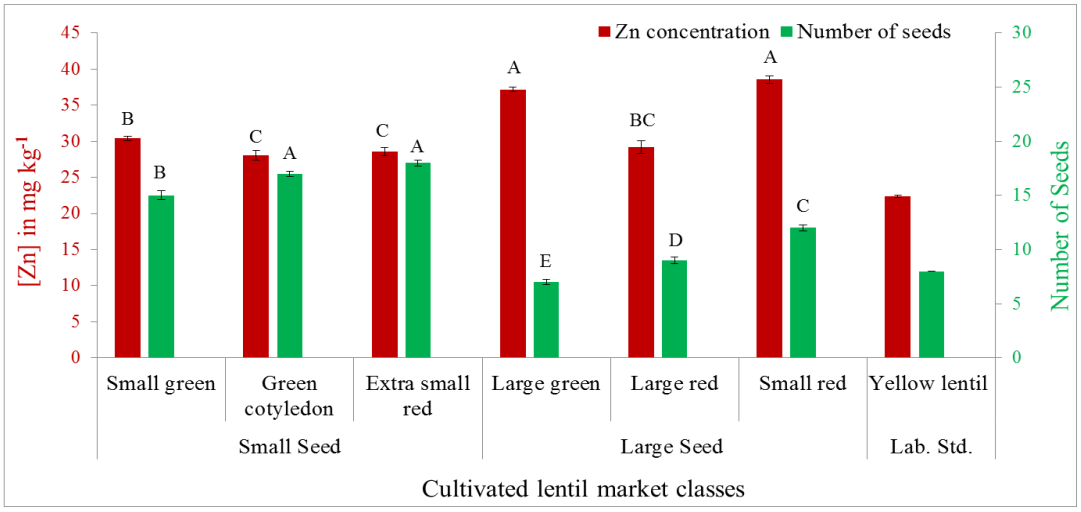

Figure 3.6. Zn concentration (primary vertical axis) and mean number of lentil seeds (secondary vertical axis) in 0.5 g of six market classes of cultivated lentil. Different letters above bars indicate significant differences in Zn concentration at $p \leq 0.05$ among different cultivated lentil market classes.

3.7 Conclusions

The method we reported here for measuring Zn concentration in whole lentil seed samples indicates accurate determination of Zn in lentil seeds by F-AAS. As little as 0.3 g of

wild and 0.5 g of cultivated lentil seeds are the most reliable sample sizes of lentil seeds for precise estimation of Zn from same seed sample. Since sample preparation does not require grinding, this procedure is rapid and simple, and therefore useful for routine analysis. In future, genotypes with contrasting Zn concentration could be used to conduct experiments for better understanding of Zn accumulation and homeostasis in lentils, and to investigate methods for developing cultivars with high Zn concentration in lentil seeds. These findings help to establish the minimum amounts of valuable and rare seed required for micronutrient analyses of seed samples of wild lentil species and their interspecific hybrids.

3.8 Acknowledgements

The authors are grateful for technical assistance provided by B. Barlow, D.D. Silva, A. Sackville from the Crop Development Centre, Department of Plant Sciences at University of Saskatchewan and B. Goetz for micronutrients analysis.

4. SEED ZINC CONCENTRATION AT THREE GROWTH STAGES IN THREE ENVIRONMENTS FOR SEVEN LENTIL SPECIES

4.1 Introduction

Genetic approaches to increasing crop productivity while enhancing micronutrient content are current goals of research programs for many food crops. Wild species of lentil are under intensive investigation as a means of increasing genetic diversity in lentil breeding for improvement of micronutrient concentration, disease resistance and other traits. Genotypic selection for improved micronutrient concentration with stable seed yield using indigenous landraces and wild genotypes are the focus of biofortification studies. Both Zn uptake and utilization in plants depend on the capability of plant genotypes. Hacisalihoglu et al. (2004) reported that Zn-efficient genotypes had higher yield capacity compared to Zn-inefficient genotypes. Efficient extraction of soil Zn might be a heritable trait and therefore selection of efficient genotypes could be used in Zn biofortification programs. Genetic gain in the development of Zn efficient lentil cultivars can be improved by introduction of new alleles from exotic wild germplasm. Positive alleles from both parents could be utilized in cultivated lentil breeding programs (Gupta & Sharma, 2007).

Wild lentil production remains difficult because of both genotypic and environment influence. Soil temperature changes influence on root development, nutrient absorption, and usage efficiencies in each growth phase of plant development (Gavito et al., 2001). Many wild lentil genotypes are highly indeterminate in nature. In Saskatoon, the months of July and August are generally considered the period for flowers and seed filling. However, Zn accumulation in seeds of wild lentil species during different stages of indeterminate plant growth has not been reported. This experiment was conducted using the 14 wild lentil genotypes from the seven *Lens* species that have been used to develop intraspecific and interspecific RIL populations at the CDC. These were grown with three replications at three environments around Saskatoon.

4.2 Hypotheses

Seed Zn concentration in *Lens* species differs in seasonal harvests.

Genotype × Harvest influences seed Zn accumulation in seeds of *Lens* species when harvested at different growth stages.

4.3 Objectives

To identify which seasonal harvest period for lentil seeds results in the highest seed Zn concentration

To determine the Genotype × Harvest timing interaction effects on Zn uptake in seeds of *Lens* species

4.4 Materials and Methods

4.4.1 Plant Material

A total of 14 genotypes (Table 4.1), including 12 wild genotypes (used as parents for developing intraspecific and interspecific RILS) and two local cultivated genotypes were selected for this study. Two genotypes from each of six wild *Lens* species were used. All were obtained from the Crop Development Centre, U of S, Saskatoon, Canada. Seed was harvested in three consecutive maturity periods. The study was conducted at three locations in Saskatoon i.e., Crop Science Field Lab (CSFL) in 2014 and CSFL and Sutherland (STH) farm in 2015. Net bags were used to avoid seed losses due to dehiscence and shattering.

Table 4.1. Lentil species and genotypes used for measurement of seed Zn concentration at three different maturity stages.

Wild lentil species and genotypes	
Lens species	Genotypes
Lens culinaris	CDC Maxim, CDC Greenstar
Lens orientalis	IG 72611, IG 72643
Lens tomentosus	PI 572390, IG 72613
Lens lamottei	IG 110810, IG110813
Lens odemensis	IG 72760, IG 72623
Lens ervoides	L01-827A, IG 72815
Lens nigricans	IG 136681, IG 116024

4.4.2 Phenotyping and Seed Zn Concentration Estimation

Mature seeds from each genotype were harvested three times during the lentil growing season (Figure 4.1). Pods were hand plucked from individual plants to avoid seed loss and seed

mixing. The first harvest was collected after 50 % pod maturity of each individual plant. After the first harvest, the second, and the third harvests occurred at 10-12 days intervals depending on the progress of pod maturation of different *Lens* species. After every harvest, net bags were tagged to protect and separate the next harvest. At each harvest all mature pods were collected, placed in paper envelopes, and then stored separately at room temperature prior to Zn concentration estimation. F-AAS analysis (described in chapter 3) was used to estimate the Zn concentration (mg kg^{-1}) of all harvested samples.

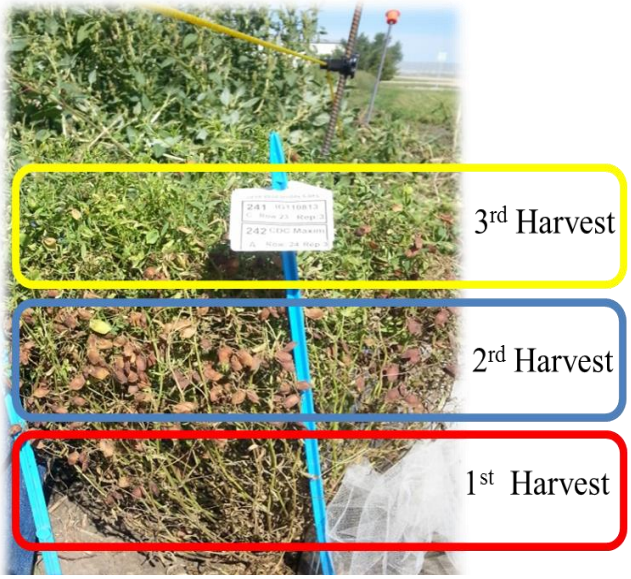

Figure 4.1. Seasonal seed harvests during the lentil growing season.

4.4.3 Statistical Analysis

The experiment was designed as a RCBD (Randomized Complete Block Design) with three replicates. Repeated statistical analyses were used with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) to study the mean difference among different seed harvests of individual genotypes. SAS Proc Mixed was used with genotype and the interaction of environment and genotype as fixed factors, and the replication was nested within location as a random factor.

The 2014 growing season was much wetter than the 2015. The statistical analysis was carried out separately (individual year) in order to study the effects of environmental factors on the Zn concentration in seeds.

4.4.4 Site Description and Environmental Conditions

Soil samples collected from different parts of each experimental site were analysed by ALS Laboratory Group Agriculture Services, Saskatoon, Canada (Table 4.2). The average monthly total precipitation (mm) and temperature of the growing seasons (May to August) of each environment are given in Table 4.3. The mean monthly temperature was similar across the three environments. However, the 2014 growing season was much wetter than 2015 in context of total precipitation reported.

Table 4.2. Soil analysis from two field locations at Saskatoon (Crop science field laboratory (CSFL) and Sutherland (STH) farm) in 2014.

Location	Depth (inches)	Texture	pH	Salinity Rating	Organic Matter (%)	DTPA-extractable [Zn] (mg kg^{-1})
CSFL	0-6	Loam	7.5	NS	3.7	2.0
STH	0-6	Clay Loam	6.6	NS	3.6	1.0

Note: ***NS***, Non-saline

Table 4.3. Monthly mean air temperature and total precipitation during the lentil growing season (May-August) in 2014-15.

Year		2014	2015
Mean temperature (^{o}C)	May	10.1	10.1
	June	14.1	17.2
	July	18.3	19.4
	August	17.9	17.4
	Overall mean	15.1	16.0
Total precipitation (mm)	May	61.1	0.4
	June	94.8	13.6
	July	44.5	84.3
	August	18.5	45.2
	Total	218.9	143.5

Note: Based on data from Environment Canada.

4.5 Results and Discussion

A wide range of variation in Zn concentration in lentil seeds was observed due to genotype. Seed Zn concentrations of the seven *Lens* species were significantly different ($p \leq 0.001$) in all three environment studied in 2014 and 2015. Seed Zn concentration among three harvests was not significantly different in three environments. For the three harvest periods and environments, Zn concentrations in seeds were significantly different only in 2014 ($p \leq 0.05$). However, the same trend did not occur in the other two environments in the 2015 growing season (Table 4.4). This may be explained by the fact that total rain fall received that was higher in 2014 than 2015. Soil Zn status in different environments affects the rate of accumulation of Zn for different plant species (Zeng et al., 2011). Seeds maturing during different growing seasons experience

difference in temperature, precipitation, and photoperiod regimes (Table 4.3). Root colonizing mycorrhiza reacts differently at different temperatures in sorghum root. Higher temperature increases root colonization with arbuscular mycorrhiza, root growth, and micronutrients uptake (Al-Karaki et al., 2004; Raju et al., 1990). Schwartz et al. (1987) observed 82% higher Zn uptake at 20^0 C compared with 10^0 C in barley. Similarly, variation in chemical and biological properties of different soils may also affect Zn uptake in lentil species. Moreover, there were differences in pH and DTPA soil status of the two locations (Table 4.2). Seed Zn concentration in different species might be attributed to the phenological and physiological differences among *Lens* species.

Table 4.4. Analysis of variance with F-values and significance levels for Zn concentration of 12 wild lentil genotypes and 2 cultivated genotypes from three environments (Crop Science Field Lab (CSFL) in 2014 and CSFL and Sutherland farm (STH) in 2015).

		F- value		
		2014	2015	2015
Effect	df	CSFL	CSFL	STH
Genotype	13	8.23^{**}	3.16^{**}	25.32^{**}
Harvest	2	0.79^{ns}	1.50^{ns}	2.03^{ns}
Genotype × Harvest	26	3.01^{*}	0.92^{ns}	0.23^{ns}

Note: ns, not significant; *, significant at $p \leq 0.05$; **, significant at $p \leq 0.001$.

In general, the second harvest tended to have the highest concentration of Zn in seeds (Figure 4.2). Zinc deficiency appears during the early growing season because of low intensity of light, and root growth increases under higher light intensity (Edwards & Kamprath, 1974). *Lens lamottei* (secondary gene pool) had the highest Zn accumulation in seeds irrespective of environments and harvest times. *L. ervoides* (tertiary gene pool) had the lowest accumulation of Zn in all tested environments followed by *L. nigricans* (quaternary gene pool). Of the remaining species, *L. lamottei* and *L. orientalis* (primary gene pool) had higher accumulation of Zn compared to most other species. *L. lamottei* had an average of 39.2% and 26.7% more seed Zn concentration than *L. ervoides* and *L. culinaris*, respectively.

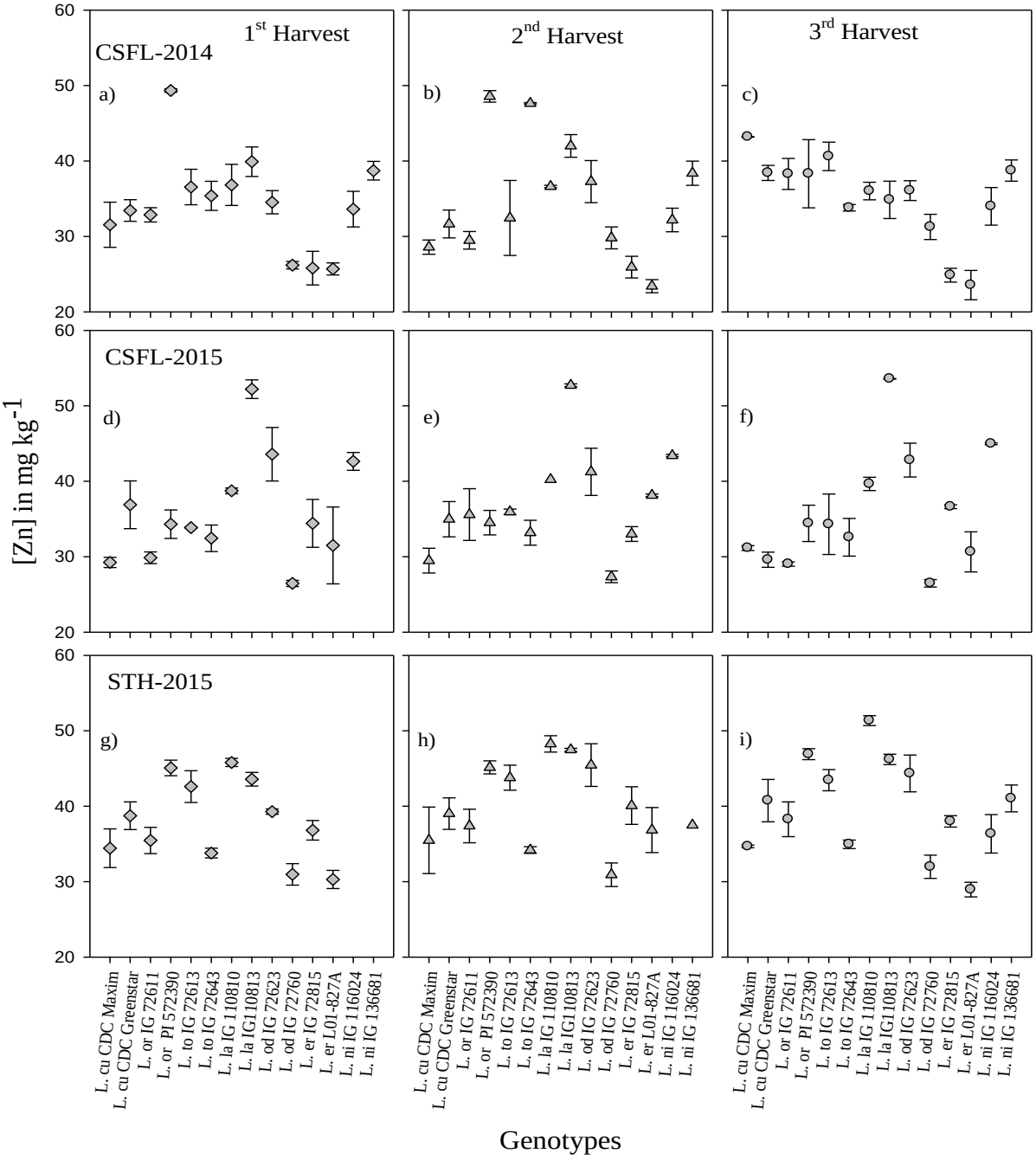

Figure 4.2. Mean zinc concentration (mg kg^{-1}) of lentil seeds harvested at three growing stages in three environments (2014-15). X and Y- axis represent the Zn concentration in seeds and genotypes from seven lentil species. Environments were Crop Science field laboratory (CSFL) - 2014 (a, b, and c), CSFL-2015 (d, e, and f), and Sutherland agriculture farm (STH) -2015 (g, h, and i). Letters indicate the mean Zn concentration in first, second, and third harvest, respectively. Error bars are standard errors of means.

Differences for Zn uptake and accumulation in *Lens* species may be because of difference in genotypic ability. In general, Zn efficient genotypes of maize accumulate higher amount of Zn than the inefficient ones (Singh et al., 2005). Zinc efficiency is influenced by root size and morphology, which varies among species (Chen et al., 2009b). Increased Zn uptake might be dependent on root surface area, root canonization by mycorrhizae, and Zn chelating phytosiderophores released from roots (Tolay et al., 2001). Accumulation of Zn was not highly influenced by the environmental factors or their interaction during the different harvest periods of the lentil plants.

Difference in seed Zn concentration in *Lens* species explained by the true genetic variability rather than the Genotype × Harvest interaction that occurred to a limited extent (only in 2014) in seeds maturing throughout the lentil growing season. *Lens lamottei* in particular could be a potential genetic resource for micronutrient improvement in cultivated lentils through interspecific breeding. It may be possible to introgress genes that influence the higher seed Zn concentration in *L. culinaris*.

5. GENOTYPE × ENVIRONMENT INTERACTION ON ZN ACCUMULATION IN SEEDS OF LENTIL INTRASPECIFIC RECOMBINANT INBRED POPULATION

5.1 Introduction

Genotype by environmental (G × E) interactions may explain the differential performance of genotypes over environments. The effect of G × E interactions may depend on the relative environmental sensitivities of the homozygous and heterozygous genotypes. These interactions are commonly observed in quantitative trait to observe the polygenic variation for the trait. The G × E interactions reduce the association between phenotypic and genotypic values. Lentil is known to have ability to grow in different environmental conditions (Saskatchewan Ministry of Agriculture, 2010). Environmental sensitivity is considered a distinct trait and is determined by mean performance of the genotypes. G × E interactions involve different physiological influences on most quantitative traits, making it more difficult to achieve progress in genetic enhancement. Temperature and photoperiod are the two major environmental factors influencing many agronomic traits of crops. Photoperiod and temperature fluctuations affect the normal growth and development of lentil plants. Knowledge of flowering time and maturity is important for lentil adaptation in all environments, especially regions like western Canada where the crop was recently introduced. Flowering time affects maturity and crop duration of many major crops in western Canada.

Soil nutrient profile is considered to be one the most influential environmental factors for micronutrient uptake studies. Available micronutrients in soils affect the genotypic capabilities for micronutrient accumulation in plants. This study focused on some of the important physiological traits associated with Zn accumulation capability in lentil seeds.

5.2 Hypothesis

Zn accumulation in lentil seeds of recombinant inbred lines from an intraspecific *L. culinaris* cross and their parents is influenced by genotype, environment, and their interactions.

5.3 Objective

To determine the Zn concentration in the lentil seeds of LR-08 population grown in different environments.

To determine the G × E interaction effects on Zn accumulation in lentil seeds of RIL population (LR-08).

5.4 Materials and Methods

5.4.1 Plant Material

The recombinant inbred line (RIL) population LR-08 was developed from a cross between *L. culinaris* accession ILL 7502 and Canadian lentil cultivar 'CDC Redberry' (Vandenberg et al., 2006) at the Crop Development Centre (CDC), University of Saskatchewan. The RIL population was developed to study micronutrient concentration and to map gene(s) associated with micronutrient content in lentil seeds. A RIL population of 120 individuals was developed by advancing F_2 plants using single seed descent (SSD) from F_2 to F_7 generation then bulking the seeds from each plant individually. Hill plots with three replicates were grown at Saskatchewan Pulse Growers (SPG) and the U of S Sutherland (STH) farms in 2014 and at STH and the Crop Science Field Laboratory (CSFL) farms in 2015. At maturity, plants of entire plot were harvested by hand pulling, dried, stored, and then each plot sample was threshed individually. Seeds were stored at room temperature for estimation of Zn concentration using F-AAS (described in chapter 3)

5.4.2 Phenotyping

The phenotypic data recorded for each of the 120 RILs and parents included days to flowering (DTF), days to maturity (DTM), plant height (PH), 1000-seed weight (TSW), and concentration of Zn ([Zn]) in seed samples. In 2014, only Zn concentration and TSW were measured at the two locations. All phenological and morphological data were recorded on individual hill plot and the mean of ten plants was used to represent each biological replication. Seed samples of every RIL were analyzed by F-AAS for estimation of Zn concentration (method described in chapter 3) in comparison with the parental genotypes. Phenotypic data (PH, DTF, DTM, TSW, and [Zn]) were collected from both locations in 2015.

5.5 Statistical Analysis

5.5.1 Phenotypic Data Analysis

Analysis of variance (ANOVA) was used to estimate the variation of Zn concentration with other measured phenotypic traits of the RIL population grown at four environments (2014-15) in Saskatoon. The 2015 growing season was much drier than 2014, although mean air temperature was similar (Table 4.3). Soil type and micronutrient profile were different among the three locations (Table 5.1). Intraspecific lentil recombinant inbred population LR-08 showed variation

for traits measured in 2015. Table 5.2 shows five measured traits (plant height, days to flowering, days to maturity, 1000 seed weight, and seed Zn concentration) based on the average of 120 RILs with parental genotypes grown at two locations in 2015.

Combined statistical analysis was used with SAS version 9.4 (SAS institute Inc., Cary, NC, USA). The seeding date of each environment was different. In order to avoid unbalanced data, phenotypic data from each year were analysed separately. The statistical analysis of phenotypic data was divided into two aspects: 1) analysis of variance for RILs evaluated in 2014 at SPG and STH, and 2) analysis of variance for RILs grown at STH and CSFL in 2015. SAS Proc Mixed procedure was used with genotype, environment and the interaction of genotype by environment as fixed factors, and replicates were nested within each environment as a random factor. Variance of RILs grown at two environments was analyzed for homogeneity analysis. SAS Proc Mixed procedure was used for genotype, environment, and their interactions as fixed factors and replicates nested within each environment was considered as random factor for the estimation of variance components. The phenotypic variance was estimated as $\sigma^2_P = \sigma^2_G + (\sigma^2_{GE} /e) + (\sigma^2_e / re)$, where σ^2_G was the estimated genotypic variance, σ^2_{GE} was the genotype-environmental interactions, σ^2_e was error variance estimate, e was the number of environments tested, and r was the number of replicates per environment. Broad-sense heritability for each trait was defined as $H^2 = \sigma^2_G / \sigma^2_P$ as described by Singh et al. (1993) and Ubayasena et al. (2010).

Table 5.1. Soil analysis from three field locations at Saskatoon (Crop science field laboratory (CSFL), Sutherland farm (STH), and Saskatchewan pulse growers farm (SPG)) in 2014.

Location	Depth (inches)	Texture	pH	Salinity Rating	Organic Matter (%)	DTPA-extractable [Zn] (mg kg^{-1})
CSFL	0-6	Loam	7.5	NS	3.7	2.0
STH	0-6	Clay Loam	6.6	NS	3.6	1.0
SPG	0-6	Loam	7.6	NS	4.0	1.5

Note: ***NS***, Non-saline.

Table 5.2. Simple statistics of LR-08 population for five traits measured at two locations (Crop Science Field Laboratory and Sutherland farm) in 2015.

Parents\Trait	PH	DTF	DTM	TSW	ZN
ILL 7502	29.0	46.5	79.2	30.9	32.7
CDC Redberry	36.2	48.2	81.2	38.4	36.6
Minimum	28.2	40.0	81.5	25.2	30.8
Maximum	41.3	52.8	96.0	41.2	47.0
Mean	34.4	45.3	87.5	33.4	37.3
Std. Dev.	2.9	2.4	2.7	3.4	2.9

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}).

5.6 Results and Discussion

5.6.1 Phenotypic Variation

Considerable phenotypic variation was observed in the evaluated traits in the RIL population and the parental genotypes. All evaluated phenotypic traits showed normal Gaussian frequency distribution patterns among RIL population (Figure 5.1-5.7), which suggests quantitative inheritance of these traits. This is similar to results found for Andean populations of common bean in which micronutrient concentration in seed was found to be quantitatively inherited (Cichy et al., 2009).

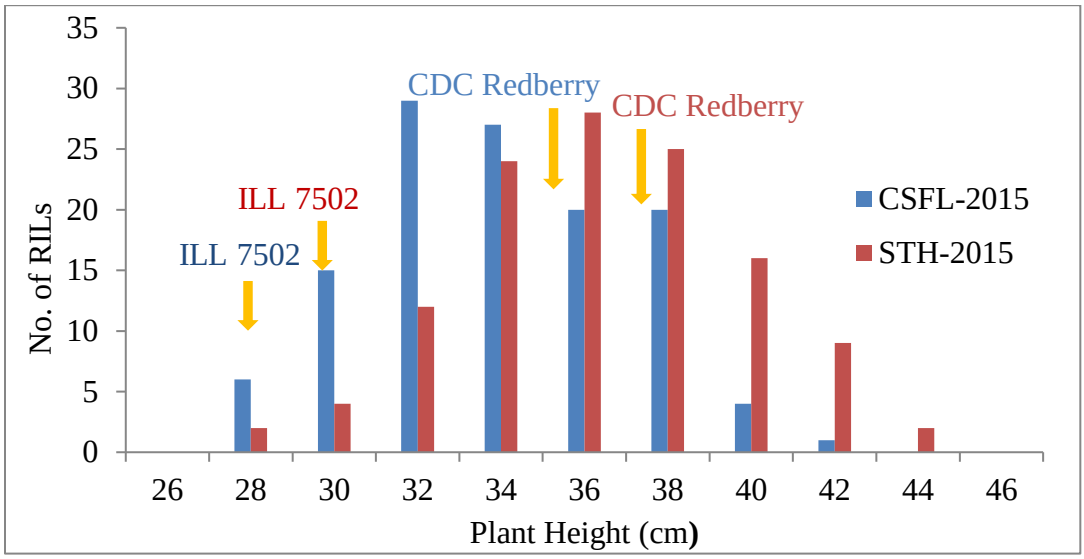

Figure 5.1. Frequency distribution of plant height (cm) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 33.3 cm; Sutherland (STH) farm: Mean of RILs = 35.6.

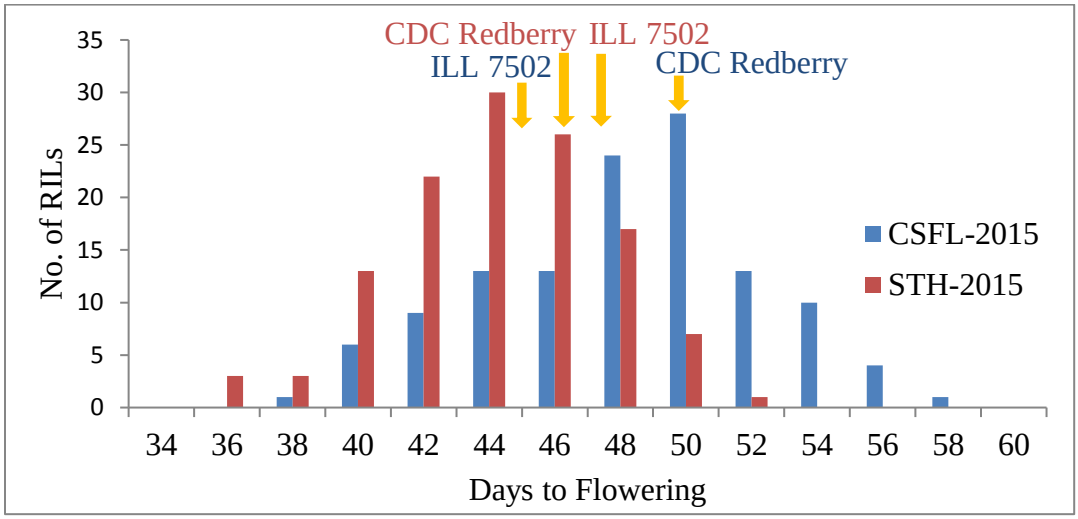

Figure 5.2. Frequency distribution of days to flowering of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 47.3; Sutherland (STH) farm: Mean of RILs = 43.4.

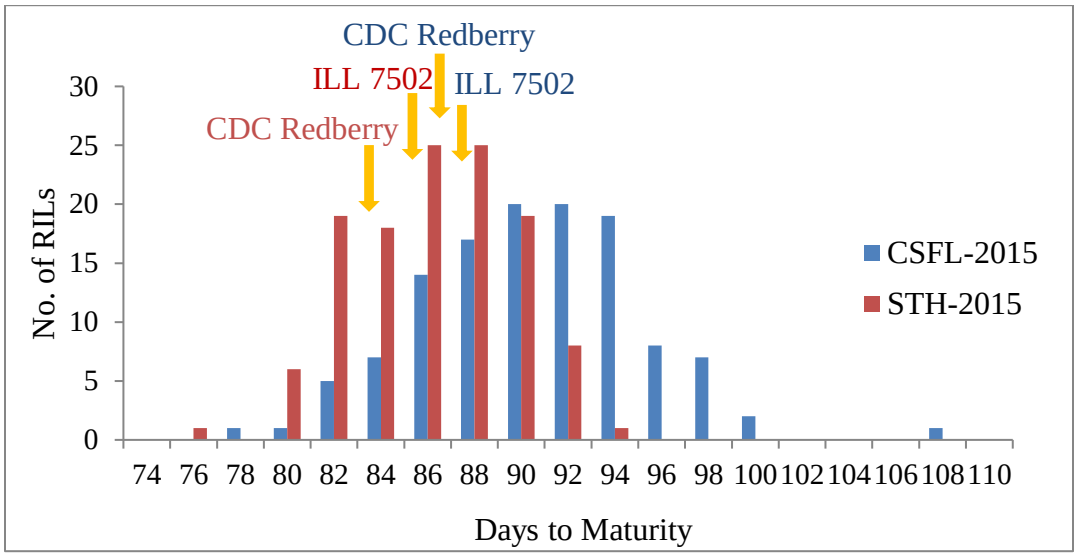

Figure 5.3. Frequency distribution of days to maturity of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 89.7; Sutherland Agricultural (STH) farm: Mean of RILs = 85.4.

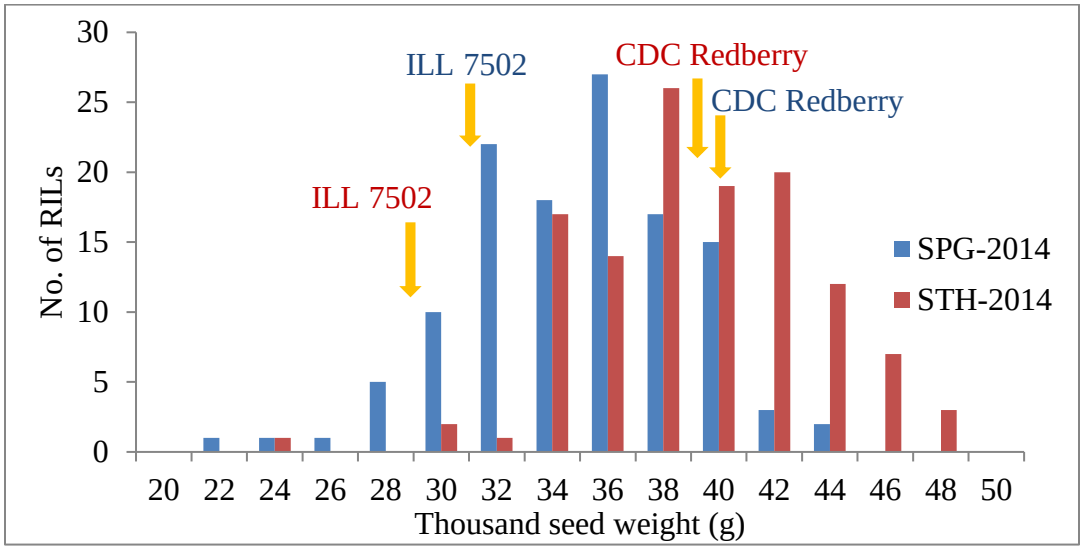

Figure 5.4. Frequency distribution of thousand seed weight (g) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2014. Saskatchewan Pulse Growers (SPG): Mean of RILs = 34.0; Sutherland (STH) farm: Mean of RILs = 38.2.

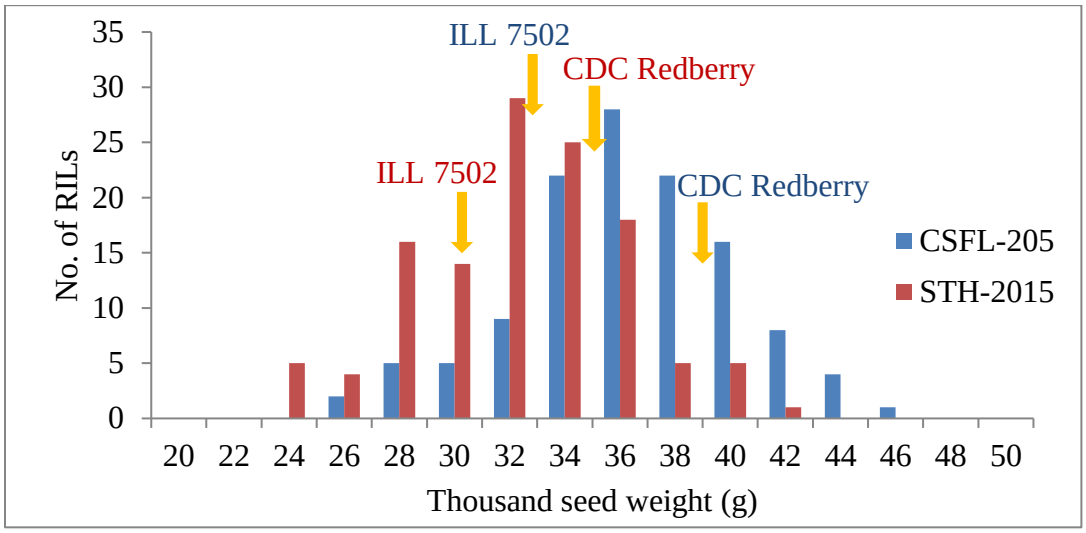

Figure 5.5. Frequency distribution of thousand seed weight (g) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 35.3; Sutherland (STH) farm: Mean of RILs = 31.4.

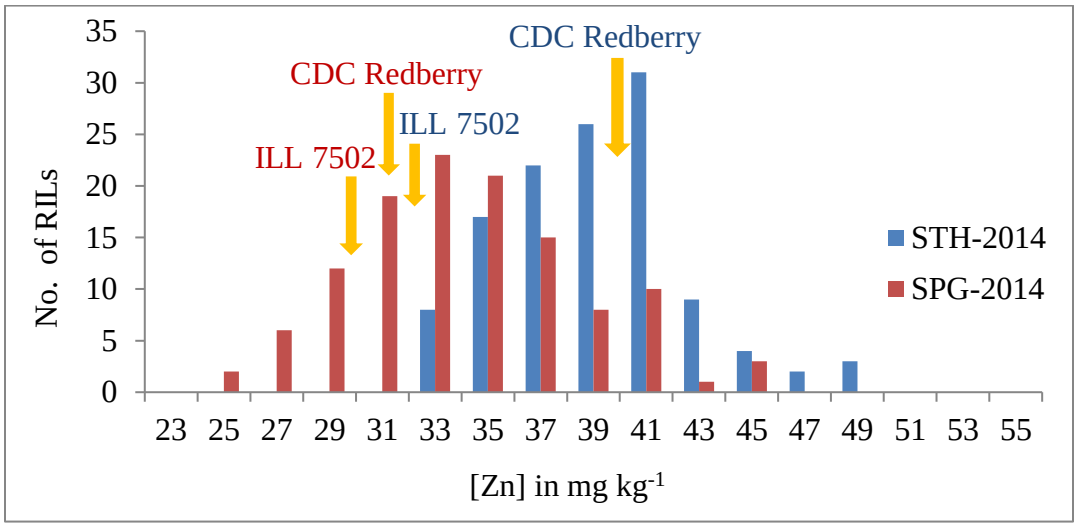

Figure 5.6. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2014. Saskatchewan Pulse Growers (SPG): Mean of RILs = 33.6; Sutherland (STH) farm: Mean of RILs = 38.5.

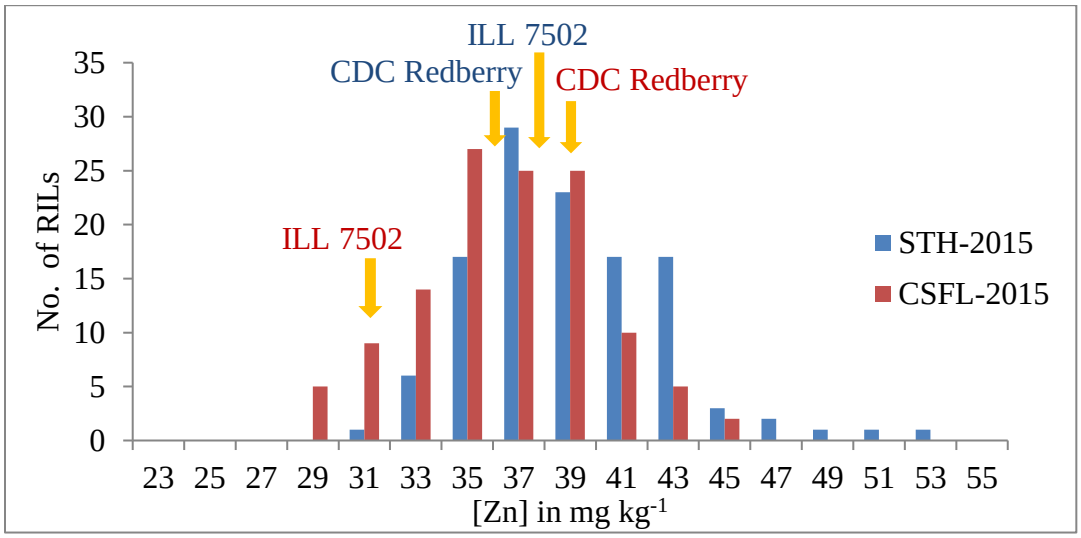

Figure 5.7. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 120 intraspecific lentil recombinant inbred lines (RILs) derived from ILL 7502/CDC Redberry (LR-08) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 35.9; Sutherland (STH) farm: Mean of RILs = 38.5.

G × E interaction effects are extremely important in the development and evaluation of plant cultivars because they reduce genotypic stability under diverse environments. Thousand seed weight were significantly affected by G×E interaction effects. Both genotype and environment individually had significant effects on Zn accumulation in lentil seeds (Table 5.3). Average seed Zn concentration was higher in 2015 compared 2014. This variation mainly may be attributable to the substantial difference in temperature and total precipitation during the growing seasons. However, significant G×E interaction effects were not observed for seed Zn concentration. This might be because environments were similar within years. This RIL population was evaluated at SPG and STH locations in 2014. There was a little difference in soil properties among the locations tested in 2014 (Table 5.1). In 2015, LR-08 population was studied at CSFL and STH. The soil status exhibited variation for DTPA extractable Zn concentration among CSFL and STH locations (Table 5.1). Except pH, other soil properties of both locations were similar. Kumar et al. (2013) reported G×E interaction effects on seed Zn concentration in lentil. However, environment had a small effect on Zn compared to the variation of Fe concentration in lentil. These environmental effects on Zn accumulation may be because of

soil type, moisture, pH, and G×E. This information provides insight for selecting specific breeding strategies in future for Zn biofortification in lentil.

Table 5.3 shows the coefficient of variation (CV) of all five traits measured at two locations in 2015. Under stress condition in 2014, seed Zn accumulation was highly variable, possibly due to impaired micronutrient absorbance through roots. The mean of each specific trait was different among environments. The analysis of variance of seed [Zn], TSW, DTF, DTM, and PH for the RIL population evaluated in 2014 and 2015 are provided in Table 5.3. Genotypes varied significantly ($p \leq 0.001$) for [Zn], TSW, and PH in 2014-15 although genotypic differences in DTF and DTM were not significantly different in 2015. Environmental effects were significant for all traits observed in 2014-15. The genotype by environment interaction effects were significant ($p \leq 0.001$) only for TSW in both 2014 and 2015.

Chakraborti et al. (2011) reported the significant G×E interaction effects for kernel Zn concentration in maize for which a significant genotypic effect was observed for seed Zn concentration. In our lentil study, this genotypic influence suggested the genetic effect on Zn accumulation in seeds. Directional selection and recombination events could in theory increase the Zn concentration substantially. Stable genotypes with contrasting seed Zn concentration could be used in lentil breeding programs to enhance genetic variability and subsequent use of QTL analysis of Zn concentration of lentil seeds.

Table 5.3. Summary of analysis of variance for plant height, days to maturity, thousand seed weight, and zinc concentration for 120 lentil recombinant inbred lines of LR-08 derived from a cross between ILL 7502/CDC Redberry evaluated at Saskatchewan Pulse Growers (SPG), Sutherland (STH) farms, and Crop Science Field Laboratory (CSFL) in 2014 and 2015.

		F-value						
		2014	2014	2015	2015	2015	2015	2015
Effect	df	[Zn]	TSW	[Zn]	TSW	DTF	DTM	PH
Environment	1	166.21*	439.59*	52.13*	869.39*	59.15*	55.85*	95.99*
Genotype	121	1.97*	10.95*	1.91*	23.30*	0.67ns	0.68 ns	5.15*
Environment × Genotype	121	1.04 ns	2.54*	1.09 ns	5.54*	0.97ns	0.91 ns	0.91ns
CV (%)		18.28	14.18	14.95	13.35	12.59	7.25	12.38

Note: ***TSW***, 1000 seed weight (g); ***DTF***, days to flowering; ***DTM***, days to maturity; ***PH***, plant height (cm); ***[Zn]***, Zn concentration (mg kg^{-1}); ns, not significant, and *, significant at $p \leq 0.001$.

5.6.2 Estimation of Variance Components and Heritability

The heritability of TSW was stable and high between the 2014 and the combined data for 2015, but varied for [Zn] in lentil seeds (Table 5.4). In 2015, [Zn], PH, and TSW showed high heritability; the highest was in PH ($H^2 = 0.82$), followed by TSW ($H^2 = 0.77$), and Zn concentration ($H^2 = 0.46$). Days to flowering and days to maturity showed moderate heritability ($H^2 = 0.21$ and 0.31, respectively). However, seed Zn concentration showed low heritability ($H^2 = 0.15$) in 2014.

Table 5.4. Estimates of variance components and broad-sense heritability of plant height, days to maturity, thousand seed weight, and zinc concentration in 120 lentil recombinant inbred line population (LR-08) derived from a cross between ILL 7502/CDC Redberry evaluated at Saskatchewan Pulse Growers and Sutherland farms in 2014 and the Crop Science Field Laboratory and Sutherland farm in 2015.

Variance component	2014	2014	2015	2015	2015	2015	2015
	[Zn]	TSW	[Zn]	TSW	DTF	DTM	PH
σ^2 Genotype	1.10	10.25	3.73	8.79	1.79	2.41	6.95
σ^2 Genotype × Environment	2.98	3.76	2.70	4.61	7.42	1.22	0.00
σ^2 Environment	28.63	7.21	17.87	2.71	18.61	28.03	8.86
σ^2 Phenotype	7.37	13.34	8.06	11.46	8.60	7.70	8.43
H^2	0.15	0.77	0.46	0.77	0.21	0.31	0.82

Note: ***TSW***, 1000 seed weight (g); ***DTF***, days to flowering; ***DTM***, days to maturity; ***PH***, plant height (cm); ***H^2***,broad-sense heritibility; ***[Zn]***, Zn concentration (mg kg^{-1}).

5.6.3 Correlations in 2015

Pearson correlation coefficients among five measured traits were estimated based on the data collected in 2015. Table 5.5 shows the correlation coefficient, mean, and standard deviation of 120 RILs at two environments in 2015. Plant height had positive significant correlation (r = 0.16, $p \leq 0.001$) with [Zn] in lentil seeds, while other traits had negative correlation. Plant height was negatively correlated with TSW (r = - 0.20, $p \leq 0.001$). Days to flowering (DTF) had significant positive correlation with days to maturity (DTM) (r = 0.95, $p \leq 0.001$) and negative correlation with [Zn] (r = - 0.20, $p \leq 0.001$). However, negative significant correlation was observed among DTF and PH (r = - 0.12). Days to maturity had a significant negative correlation

with Zn concentration (r = - 0.19, $p \leq 0.001$) and positive non-significant correlation with TSW (r = 0.01). Thousand seed weight had significant negative correlation with [Zn] (r = - 0.21, $p \leq 0.001$). Overall, correlation analysis revealed the negative correlation between [Zn] and days to flower, days to maturity, and thousand seed weight. Recombinant inbred lines with higher seed [Zn] took longer time for flowering and maturity. Correlations among these measured traits with seed [Zn] of lentil demonstrate the importance of effective selection for cultivar development of higher Zn concentration.

Table 5.5. Pearson correlation coefficients for plant height, days to flowering, days to Maturity, 1000 seed weight, and seed Zn concentration for 120 RILs of LR-08 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015.

	PH	DTF	DTM	TSW	[ZN]
PH (34.4±4.3)	1	-0.12*	-0.09^{ns}	-0.20**	0.16**
DTF (44.7±5.6)		1	0.95**	-0.01^{ns}	-0.20**
DTM (86.6±6.3)			1	0.02^{ns}	-0.19**
TSW (33.3±4.4)				1	-0.21**
[ZN] (37.4±5.6)					1

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}); ns, not significant; *, significant at $p \leq 0.01$; **, indicates significant correlation at $p \leq 0.001$, and numbers in the brackets represents the mean and standard error values for the traits were averaged over three replications at two locations.

5.6.4 Selection of RILs from the Intraspecific RIL populations

The RILs with the ten highest and lowest seed [Zn] at two tested environments in 2015 were compared (Table 5.6). RILs with the highest seed [Zn] tended to be late flowering, late maturing and had higher plant height compared to the RILs of lowest seed [Zn]. The same was observed for the parental genotypes. The two selected groups of RILs were significantly different ($p \leq 0.05$) for all traits observed except for TSW.

Table 5.6. Comparison of 10 selected RILs with the highest and lowest seed mean Zn concentration at two locations.

RIL Parents	PH	DTF	DTM	TSW	[Zn]
ILL 7502	29.0	46.5	79.2	30.9	32.7
CDC Redberry	36.2	48.2	81.2	38.4	36.6
Mean of RIL population	34.5	45.4	79.6	34.7	36.6
RILs with lowest seed [Zn]					
LR-8-21	33.5	44.0	89.5	35.7	31.0
LR-8-47	32.8	41.8	83.0	31.6	31.0
LR-8-97	32.9	55.5	87.3	33.3	31.8
LR-8-28	34.2	41.5	85.3	30.1	32.3
LR-8-34	28.3	40.0	81.3	34.4	32.3
LR-8-109	34.2	46.5	85.3	41.2	32.3
LR-8-90	34.0	41.7	86.2	32.0	32.8
LR-8-180	31.5	45.5	87.7	29.5	32.8
LR-8-101	34.3	42.8	83.2	35.4	33.0
LR-8-26	30.7	42.8	84.3	34.3	33.3
Mean	32.6	44.2	85.3	33.7	32.3
RILs of highest seed [Zn]					
LR-8-31	36.3	47.0	88.5	32.7	41.0
LR-8-189	39.5	46.2	88.8	36.6	41.1
LR-8-61	36.5	51.3	89.2	36.7	41.3
LR-8-179	35.7	42.8	89.7	33.4	41.5
LR-8-73	37.7	45.7	86.8	29.2	42.0
LR-8-96	37.3	47.3	90.7	36.7	42.0
LR-8-8	37.0	47.7	85.7	30.0	42.1
LR-8-84	36.5	46.3	87.7	35.5	42.5
LR-8-42	32.7	46.2	88.2	26.1	43.0
LR-8-3	36.0	50.3	96.0	30.6	45.4
Mean	36.5	47.1	89.1	32.7	42.2
P > |t|	0.0002*	0.0872*	0.0046*	0.5351ns	<.0001*

Note: ***TSW***, 1000 seeds weight (g); ***DTF***, days to flowering; ***DTM***, days to maturity; ***PH***, plant height (cm); ***[Zn]***, Zn concentration (mg kg^{-1}); ns, not significant; *, significant at $p \leq 0.05$.

Information from this experiment could be verified again by testing these RILs in more diverse environments. Micronutrient uptake and accumulation traits in plants were also observed to be variable in other experiments (Appendix A). Zinc efficient beans accumulated higher [Zn] in seeds compared to Zn-inefficient genotypes grown in same environment and year (Gregorio, 2002).Seed [Zn] could be improved in lentil by selective breeding. Screening for genetic variability for increased seed [Zn] can be considered the first step in plant breeding For example, the seed [Zn] in RILs of LR-08 ranged from 31- 45 mg kg^{-1} (Table 5.6). These selected genotypes could serve as key donors as parents for use in lentil breeding for higher seed [Zn] in Canada.

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

In many developing countries in South Asia lentil is part of the daily diet, representing an important source of micronutrients. Lentil breeding in Canada has focused on improving cultivars with high yield, disease resistance, and micronutrient quality to meet perceived international market demand. The development of lentil cultivars with higher seed [Zn] across a wide range of environments is the ultimate goal of the lentil breeding program. The objectives of this series of experiments were:1) to determine the minimum amount of sample size require for precise estimation of [Zn] in lentil seeds, 2) to determine if seed [Zn] differs in seasonal harvests of all *Lens* species, and 3) to evaluate the genotype by environmental interaction effects on seed [Zn] of lentil intraspecific recombinant inbred population. These objectives were met in this research studies.

F-AAS provides reliable and precise estimation of [Zn] for whole lentil seeds. A simple and economic method for the determination of [Zn] in lentil seeds of all *Lens* species by F-AAS was modified and validated. The procedure described in chapter 3 is very easy for routine analyses of a large number of samples within a short time, while minimizing the requirement of seed sample size and number of seeds. This is especially important for seed analysis of wild lentils, which are difficult to collect and reproduce. The results from this experiment confirmed the minimum weight and number of whole lentil seed sample sizes required for precise and reproducible estimation of [Zn] by F-AAS. Seed sample of 0.3 g of wild and 0.5 g of cultivated lentil seeds were identified as the minimum sample size for accurate and precise estimation of seed [Zn]. Sample sizes less than 0.3 g always had inconsistency in analysis of seed [Zn]. This might be because of over digestion of smaller seed sample size with higher digestion matrix or recommended analytical parameter set up of F-AAS. For cultivated lentil genotypes 0.1 and 0.3 g sample sizes showed inconsistency in seed [Zn] estimation. This might be explained by the seed size of cultivated lentils which are 10 to 15 fold bigger than wild lentil genotypes. This information is of value for reducing the amount of seed required for analysis of rare and valuable lentil genotypes that may contribute to genetic improvement.

The phenotypic expression is the cumulative effects of genotypic value and the environmental effect. Different environments affect the level of performance of different

genotypes. During the evaluation and selection phases of genotypes in breeding program, genotype by environmental interactions are important baseline characteristics that can influence the development of a breeding strategy. Quantitative traits are considerably affected by the influences of environmental differences. These had significant interaction effects on Zn accumulation in seeds of *Lens* species. Results in chapter 4 showed the seed harvested from the Sutherland farm always had the highest accumulation of Zn. This might be because of higher soil pH (< 7) of Sutherland farm location (Table 4.2). Seeds that mature during late development stage of plant showed higher Zn accumulation. Seeds of *L. lamottei* were observed to have genetic ability for higher accumulation of Zn, whereas *L. ervoides* had the lowest. Zinc efficient genotypes possess extensive root systems which help in higher Zn uptake (Cakmak & Marschner, 1988). This needs to be evaluated for the wild *Lens* species as a baseline for developing a breeding strategy for higher Zn accumulation. Wild *Lens* genotypes of diverse origin had significant variation with respect to agronomic and morphological characteristics (Singh et al., 2014). Promising genotypes with high seed [Zn] from *L. lamottei* and *L. orientalis* could be exploited for broadening the genetic base for biofortification of cultivated lentils. The *Lens* core collections from different gene banks, which represent about 70% of the genetic diversity of the whole sample set (Wang et al., 2006) could be screened to identify material with higher seed [Zn] – these may have more extensive root systems.

Chapter 5 results explained the potential basis for breeding strategies for increased seed [Zn] in relation to other phenotypic traits used to evaluate the effect of G×E interactions in 120 individual RILs of intraspecific inbred lentil population LR-08. The effect of G×E interactions on seed Zn concentration were also studied in other two interspecific RILs population (LR-26 & 59) in 2014-15 which are described in Appendix B and C. A significant effect of genotype was observed for seed [Zn] in two consecutive years. Significant effect of environment for seed [Zn] along with all other measured traits suggests that environment does play important role on zinc accumulation. Overall, higher [Zn] in lentil seeds was observed in 2015 compared with 2014, explainable by the difference in growing season precipitation and temperature. In 2014, both locations received more precipitation than in 2015. It may be necessary to consider carefully in the breeding strategy, how many environments would be sufficient for effective selection. Nutritional traits are generally stable across environments, despite some reported significant interaction effects of G × E on Zn accumulation in extreme environments (Gregorio, 2002).

Continuous distribution of seed [Zn] indicates the quantitative nature of inheritance. In this study, similar quantitative nature of inheritance was observed for other measured traits. These results demonstrated the polygenic control for all traits (seed Zn concentration, thousand seed weight, plant height, days to flowering, and days to maturity).

Transgressive phenotypes were observed in RIL population when compared to parental genotypes. This could be due to the large effect of environment compared with effects of genotype, year, and their interaction with intraspecific recombinant inbred population (Table 5.3). The observed transgressive segregation for [Zn] in lentil seeds and other traits could be due to the additive effects contributed by the parental genotypes to the segregated populations. Average seed [Zn] in 120 RILs population was significantly different. Non-significant interaction effects on seed [Zn] suggest the genotypic stability of performance. This might be due to the lack of sufficient diversity in soil status. However significant interaction effects of environment and year were reported for [Zn] in rice and environmental effect was much higher (60 %) than the genotypic effect (Norton et al., 2004; Diapari et al., 2015). Swarto et al. (2011) observed that seed [Zn] may be influenced by the environmental factors for example, soil Zn status of different locations.

Spatial distribution of Zn is highly variable across Saskatchewan soils (Singh, 1986). The North-east region of Saskatchewan has comparatively higher soil Zn compared to south and south-central Saskatchewan. Using these environments, this intraspecific population could be tested in diverse locations in Saskatchewan to gain deeper understanding of the G×E interaction effects on Zn accumulation in lentil seeds. Anderson, (2015) reported non-significant effect on lentil grain yield and seed [Zn] after application of $ZnSO_4$ fertilizer in diverse locations in Saskatchewan.

In the current study, positive correlation was observed between seed [Zn] and plant height. Plant height index has already been established as an effective selection tool for yield and lodging resistance for lentil breeding in developed countries. Upright habit is essential for mechanical harvest and maintaining product quality. Diverse field trials would provide a better understanding of the heritability of Zn content in lentil.

In other crops, it has been suggested that F_8 RILS could be used for genetic mapping genes associated with increased [Zn]. Cyclical recurrent selection breeding strategies could be used to develop Zn-efficient lentil cultivars. Zinc uptake efficiency from soil and accumulation

in seed could be achievable with higher yield by plant breeding. At some point in future, as awareness of Zn deficiency increases among consumers, lentil growers may be able to achieve higher profits by adopting cultivars of higher Zn dense seeds with higher yield, especially in organic farming systems.

Mapping of genes could be an important approach in developing marker-assisted selection breeding. This selection technique is becoming more reliable, more rapid and less costly. Marker-assisted selection breeding could be the most cost effective and rapid strategy for selecting two to three traits at a time. A lentil association mapping (LAM) panel (Appendix A) has been developed from diverse lentil genotypes including landraces, breeding lines, and cultivars origination from diverse environment across the world. A total of 1150 SNP markers were used for association analysis for seed [Zn] with 138 lentil genotypes tested in four environments in 2013 and 2014 (Appendix A). Three SNP markers were identified to be significantly associated with [Zn] in lentil seeds. Heritability of the seed [Zn] was moderate and stable. Backcross breeding strategy could be followed for improvement of seed [Zn] in elite lentil cultivar. Screening of lentil cultivars and RIL populations with SNP markers is the most reliable way for identification of alleles linked with polygenic traits. Breeding for zinc-efficient genotypes than can more effectively function under Zn deficient condition is an effective and sustainable solution.

6.2 Conclusions

Results from this research studies identified the minimum weight and number of whole lentil seed sample sizes for both wild and cultivated lentils when these are required for precise and reproducible estimation of [Zn] by F-AAS. Seeds mature during late development stage of plant showed higher Zn accumulation. Seeds of *Lens lamottei* were observed to have ability to accumulate higher Zn whereas *Lens ervoides* had the lowest. Genotype and environmental effects were greater for intraspecific RILs compared to environment and genotype interaction effects. Seed [Zn] in lentil was observed to be quantitatively inherited. Finally, these initiatives can contribute to the further understanding of genetic control of Zn content in lentil. Success in genetic biofortification of lentil with higher Zn content could substantially enhance sustainable micronutrient intake for humans.

6.3 Future Research

Recent studies have strongly suggested the possibilities of genetic improvement of [Zn] lentils. For breeding purposes, further screening could be carried out including more diverse set of wild relatives, elite landraces, breeding lines, and commercial cultivars that are more adaptable for different geographical regions of lentil cultivation across the world.

To overcome the soil heterogeneity, Zn efficient genotypes could be screened under greenhouse condition in pots which is easier, fast, and cost-effective than the field condition.

Genotype × Environment interaction effects on seed Zn accumulation in intraspecific RIL population can be tested in a wider range of environments combined with a range of soil type, photoperiod, and temperature regimes.

The SNP markers reported for [Zn] (Appendix A) in lentil seeds are likely to be useful for selecting favorable lines by marker assisted breeding. Selected RILs of contrasting Zn content from this research could be studied further in diverse environmental conditions for understanding the inheritance of Zn bioavailability and yield trial.

These highly polymorphic SNP markers (Appendix A) associated with [Zn] could be effective tools for transgressive segregation selection from breeding populations and genetically diverse lentil genotypes with higher Zn content could be efficiently used in Zn biofortification of lentil.

The identification of genes or markers tightly linked to gene complex and elucidation of their role in Zn accumulation of lentil seeds could be facilitated from the sequence information of identified SNP markers in this research.

Further studies could be continued for the identification of Zn-controlling genes found on lentil chromosome 3, 2, and 1. Prior knowledge of genetic markers is very important for efficient and successful lentil breeding. Lentil genetic improvement programs could be enhanced up by using the molecular marker for introgression and recurrent selection. Incorporation of putative genes responsible for Zn uptake and accumulation into adapted genotypes is the desired target for biofortification studies.

7. REFERENCES

Abarca, A., Canfranc, E., Sierra, I., & Marina, M. (2001). A validated flame AAS method for determining magnesium in a multivitamin pharmaceutical preparation. *Journal of Pharmaceutical and Biomedical Analysis, 25*(5), 941-945.

Abbo, S., Ladizinsky, G., & Weeden, N. F. (1991). Genetic analysis and linkage study of seed weight in lentil. *Euphytica*, *58*(3), 259-266.

Agrama, H., Eizenga, G., & Yan, W. (2007). Association mapping of yield and its components in rice cultivars. *Molecular Breeding, 19*(4), 341-356.

Alloway, B. J. (2008). Micronutrient deficiencies in global crop production. *Springer Science & Business Media*.

Alloway, B. J. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environmental Geochemistry and Health, 31*(5), 537-548.

Al-Karaki, G., McMichael, B. Z. A. K. J., & Zak, J. (2004). Field response of wheat to arbuscular mycorrhizal fungi and drought stress. *Mycorrhiza*, *14*(4), 263-269.

Anderson, S. (2015). Zinc fertilization of lentil in Saskatchewan to increase yield and grain zinc content. M.Sc. Thesis. *University of Saskatchewan*, Saskatchewan.

Arumuganathan, K., & Earle, E. (1991). Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter, 9*(3), 208-218.

Barulina, E. (1930). The lentils of the USSR and other countries. *Bulletin of Applied Botany and Plant Breeding, 40*, 1-319.

Bauer, A., & Lindsay, W. L. (1965). The effect of soil temperature on the availability of indigenous soil zinc. *Soil Science Society of America Journal, 29*(4), 413-416.

Benton, J. J. (2002).Management of crops, soils and their fertility. *Agronomic handbook*, CRC press.

Blair, M. W., Cortés, A. J., Penmetsa, R. V., Farmer, A., Carrasquilla-Garcia, N., & Cook, D. R. (2013). A high-throughput SNP marker system for parental polymorphism screening, and diversity analysis in common bean (Phaseolus vulgaris L.). *Theoretical and Applied Genetics, 126*(2), 535-548.

Bouis, H. E., & Welch, R. M. (2010). Biofortification-a sustainable agricultural strategy for

reducing micronutrient malnutrition in the global south. *Crop Science, 50*(Supplement1), S-20-S-32.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics, 23*(19), 2633-2635.

Breseghello, F., & Sorrells, M. E. (2006). Association mapping of kernel size and milling quality in wheat (Triticum aestivum L.) cultivars. *Genetics, 172*(2), 1165-1177.

Bundock, P. C., Eliott, F. G., Ablett, G., Benson, A. D., Casu, R. E., Aitken, K. S., & Henry, R. J. (2009). Targeted single nucleotide polymorphism (SNP) discovery in a highly polyploid plant species using 454 sequencing. *Plant Biotechnology Journal, 7*(4), 347-354.

Cabrera, C., Lloris, F., Gimenez, R., Olalla, M., & Lopez, M. C. (2003). Mineral content in legumes and nuts: contribution to the Spanish dietary intake. *Science of the Total Environment, 308*(1), 1-14.

Cakmak, I., & Marschner, H. (1988). Increase in membrane permeability and exudation in roots of zinc deficient plants. *Journal of Plant Physiology, 132*(3), 356-361.

Çakmak, İ., Torun, A., Millet, E., Feldman, M., Fahima, T., Korol, A., . . . Özkan, H. (2004). Triticum dicoccoides: an important genetic resource for increasing zinc and iron concentration in modern cultivated wheat. *Soil Science and Plant Nutrition, 50*(7), 1047-1054.

Canadian Food Inspection Agency. (2016). The Biology of Lens culinaris Medikus (Lentil). Retrieved May 16, 2016, from http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/lens-culinaris-medikus-lentil-/eng/1330978380871/1330978449837.

Chakraborti, M., Prasanna, B. M., Hossain, F., Mazumdar, S., Singh, A. M., Guleria, S., & Gupta, H. S. (2011). Identification of kernel iron-and zinc-rich maize inbreds and analysis of genetic diversity using microsatellite markers. *Journal of plant biochemistry and biotechnology, 20*(2), 224-233.

Chen, W. R., He, Z. L., Yang, X. E., & Feng, Y. (2009b). Zinc efficiency is correlated with root morphology, ultrastructure, and antioxidative enzymes in rice. *Journal of Plant*

Nutrition, 32(2), 287-305.

Choi, I.-Y., Hyten, D. L., Matukumalli, L. K., Song, Q., Chaky, J. M., Quigley, C. V., . . . Yoon, M.-S. (2007). A soybean transcript map: gene distribution, haplotype and single-nucleotide polymorphism analysis. *Genetics, 176*(1), 685-696.

Churchill, G. A., Airey, D. C., Allayee, H., Angel, J. M., Attie, A. D., Beatty, J., . . . Berrettini, W. (2004). The Collaborative Cross, a community resource for the genetic analysis of complex traits. *Nature Genetics, 36*(11), 1133-1137.

Cichy, K. A., Caldas, G. V., Snapp, S. S., & Blair, M. W. (2009). QTL analysis of seed iron, zinc, and phosphorus levels in an Andean bean population. *Crop Science, 49*(5), 1742-1750.

Colangelo, E. P., & Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Current opinion in plant biology*, *9*(3), 322-330.

Diapari, M., Sindhu, A., Bett, K., Deokar, A., Warkentin, T. D., & Tar'an, B. (2014). Genetic diversity and association mapping of iron and zinc concentrations in chickpea (Cicer arietinum L.). *Genome*, *57*(8), 459-468.

Diapari, M., Sindhu, A., Warkentin, T. D., Bett, K., & Tar'an, B. (2015). Population structure and marker-trait association studies of iron, zinc and selenium concentrations in seed of field pea (Pisum sativum L.). *Molecular Breeding*, *35*(1), 1-14.

Doyle, J. J. (1990). Isolation of plant DNA from fresh tissue. *Focus, 12*, 13-15.

Edwards, J. H., & Kamprath, E. J. (1974). Zinc accumulation by corn seedlings as influenced by phosphorus, temperature, and light intensity. *Agronomy Journal*, *66*(4), 479-482.

Environment Canada. (2013). Climate trends and variation bulletin-2013 annual. Environment Canada Catalogue no: En81-23E-PDF.

Environment Canada. (2014). Climate trends and variation bulletin-summer 2014. Environment Canada Catalogue no: En81-23E-PDF.

Environment Canada. (2015). Climate trends and variation bulletin-summer 2015. Environment Canada Catalogue no: En81-23E-PDF.

Erskine, W., Williams, P. C., & Nakkoul, H. (1985). Genetic and environmental variation in the seed size, protein, yield, and cooking quality of lentils. *Field Crops Research, 12*, 153-

161.

FAOSTAT. (2015). Food and Agriculture Organization of the United Nations. Available online at: http://faostat.fao.org/.

FAOSTAT. (2015). Food and Agriculture Organization of the United Nations. Retrieved May 16, 2016, from http://faostat3.fao.org/browse/Q/QC/E.

Fedoruk, M. J., Vandenberg, A., & Bett, K. E. (2013). Quantitative trait loci analysis of seed quality characteristics in lentil using single nucleotide polymorphism markers. *The Plant Genome*, *6*(3).

Ferguson, M. E., Maxted, N., Slageren, M. V., & Robertson, L. D. (2000). A re- assessment of the taxonomy of Lens Mill. (Leguminosae, Papilionoideae, Vicieae). *Botanical Journal of the Linnean Society*, *133*(1), 41-59.

Ferguson, M. F., and L. D. Robertson. (1999). Morphological and phonological variation in the wild relatives of lentil. *Genetic Resources and Crop Evolution, 46*, 3-10.

Fiala, J. V., Tullu, A., Banniza, S., Séguin-Swartz, G., & Vandenberg, A. (2009). Interspecies Transfer of Resistance to Anthracnose in Lentil (Medic.). *Crop Science*, *49*(3), 825-830.

Flint-Garcia, S. A., Thornsberry, J. M., & IV, B. (2003). Structure of linkage disequilibrium in plants. *Annual Review of Plant Biology*, *54*(1), 357-374.

Flint- Garcia, S. A., Thuillet, A. C., Yu, J., Pressoir, G., Romero, S. M., Mitchell, S. E., . . . Buckler, E. S. (2005). Maize association population: a high- resolution platform for quantitative trait locus dissection. *The Plant Journal*, *44*(6), 1054-1064.

Foster, J. T., Allan, G. J., Chan, A. P., Rabinowicz, P. D., Ravel, J., Jackson, P. J., & Keim, P. (2010). Single nucleotide polymorphisms for assessing genetic diversity in castor bean (Ricinus communis). *BMC Plant Biology*, *10*(1), 1.

Galeano, C. H., Cortés, A. J., Fernández, A. C., Soler, Á., Franco-Herrera, N., Makunde, G., . . . Blair, M. W. (2012). Gene-based single nucleotide polymorphism markers for genetic and association mapping in common bean. *BMC Genetics*, *13*(1), 1.

Gan, Y., Hamel, C., O'Donovan, J. T., Cutforth, H., Zentner, R. P., Campbell, C. A., . . . Poppy, L. (2015). Diversifying crop rotations with pulses enhances system productivity. *Scientific Reports*, *5*.

Gavito, M. E., Curtis, P. S., Mikkelsen, T. N., & Jakobsen, I. (2001). Interactive effects of soil temperature, atmospheric carbon dioxide and soil N on root development, biomass and nutrient uptake of winter wheat during vegetative growth. *Journal of Experimental Botany*, *52*(362), 1913-1923.

Genc, Y., McDonald, G., & Graham, R. (2002). A soil-based method to screen for zinc efficiency in seedlings and its ability to predict yield responses to zinc deficiency in mature plants. *Crop and Pasture Science*, *53*(4), 409-421.

Ghaedi, M., Mortazavi, K., Montazerozohori, M., Shokrollahi, A., & Soylak, M. (2013). Flame atomic absorption spectrometric (FAAS) determination of copper, iron and zinc in food samples after solid-phase extraction on Schiff base-modified duolite XAD 761. *Materials Science and Engineering: C*, *33*(4), 2338-2344.

Gregorio, G. B. (2002). Progress in breeding for trace minerals in staple crops. *The Journal of nutrition*, *132*(3), 500S-502S.

Gujaria, N., Kumar, A., Dauthal, P., Dubey, A., Hiremath, P., Prakash, A. B., . . . Gaur, P. M. (2011). Development and use of genic molecular markers (GMMs) for construction of a transcript map of chickpea (Cicer arietinum L.). *Theoretical and Applied Genetics*, *122*(8), 1577-1589.

Gupta, D., & Sharma, S. (2007). Widening the gene pool of cultivated lentils through introgression of alien chromatin from wild Lens subspecies. *Plant Breeding*, *126*(1), 58-61.

Gupta, P., Rustgi, S., & Mir, R. (2008). Array-based high-throughput DNA markers for crop improvement. *Heredity*, *101*(1), 5-18.

Hacisalihoglu, G., Ozturk, L., Cakmak, I., Welch, R. M., & Kochian, L. (2004). Genotypic variation in common bean in response to zinc deficiency in calcareous soil. *Plant and Soil*, *259*(1-2), 71-83.

Hafeez, B., Khanif, Y., & Saleem, M. (2013). Role of zinc in plant nutrition-a review. *American Journal of Experimental Agriculture*, *3*(2), 374.

Hotz, C., & Brown, K. H. (2004). Assessment of the risk of zinc deficiency in populations and options for its control. *International Nutrition Foundation:* for UNU.

Hussain, S., Maqsood, M., & Miller, L. (2011). Bioavailable zinc in grains of bread wheat varieties of Pakistan. *Cereal Research Communications, 40*(1), 62-73.

Hyten, D. L., Choi, I.-Y., Song, Q., Specht, J. E., Carter, T. E., Shoemaker, R. C., . . . Cregan, P. B. (2010). A high density integrated genetic linkage map of soybean and the development of a 1536 universal soy linkage panel for quantitative trait locus mapping. *Crop Science, 50*(3), 960-968.

Jameson, S. (1993). Zinc status in pregnancy: the effect of zinc therapy on perinatal mortality, prematurity, and placental ablation. *Annals of the New York Academy of Sciences*, *678*(1), 178-192.

Jun, T.-H., Van, K., Kim, M. Y., Lee, S.-H., & Walker, D. R. (2008). Association analysis using SSR markers to find QTL for seed protein content in soybean. *Euphytica, 162*(2), 179-191.

Kannenberg, L., & Falk, D. (1995). Models for activation of plant genetic resources for crop breeding programs. *Canadian Journal of Plant Science, 75*(1), 45-53.

Karaköy, T., Erdem, H., Baloch, F. S., Toklu, F., Eker, S., Kilian, B., & Özkan, H. (2012). Diversity of macro-and micronutrients in the seeds of lentil landraces. *The Scientific World Journal*, 2012.

Khazaei, H., Caron, C. T., Fedoruk, M., Diapari, M., Vandenberg, A., Coyne, C. J., ... & Bett, K. E. (2016). Genetic Diversity of Cultivated Lentil (Lens culinaris Medik.) and Its Relation to the World's Agro-ecological Zones. *Frontiers in Plant Science*, *7*.

Kumar, H., Dikshit, H. K., Singh, A. M., Singh, D., Kumari, J., Singh, A., & Kumar, D. (2013). Characterization of elite lentil genotypes for seed iron and zinc concentration and genotype x environment interaction studies. *Indian Journal of Genetics and Plant Breeding*, *73*(2), 169-176.

Lander, E. S., & Schork, N. J. (1994). Genetic dissection of complex traits. *Science*, *265*(5181), 2037-2048.

Lansdown, A. B., Mirastschijski, U., Stubbs, N., Scanlon, E., & Ågren, M. S. (2007). Zinc in wound healing: theoretical, experimental, and clinical aspects. *Wound Repair and Regeneration*, *15*(1), 2-16.

Lintschinger, J., Fuchs, N., Moser, J., Kuehnelt, D., & Goessler, W. (2000). Selenium-enriched sprouts. A raw material for fortified cereal-based diets. *Journal of Agricultural and Food Chemistry*, *48*(11), 5362-5368.

Lopez-Millan, A. F., Ellis, D. R., & Grusak, M. A. (2004). Identification and characterization of several new members of the ZIP family of metal ion transporters in Medicago truncatula. *Plant molecular biology, 54*(4), 583-596.

Letunic, I., & Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic acids research*, gkr201.

Mantovi, P., Bonazzi, G., Maestri, E., & Marmiroli, N. (2003). Accumulation of copper and zinc from liquid manure in agricultural soils and crop plants. *Plant and Soil, 250*(2), 249-257.

Marschner, H. (2011). *Marschner's Mineral Nutrition of Higher Plants*: Academic press.

Norton, G. J., Douglas, A., Lahner, B., Yakubova, E., Guerinot, M. L., Pinson, S. R., ... & Islam, M. R. (2014). Genome wide association mapping of grain arsenic, copper, molybdenum and zinc in rice (Oryza sativa L.) grown at four international field sites. *PLoS One*, *9*(2), e89685.

Ortiz-Monasterio, J., Palacios-Rojas, N., Meng, E., Pixley, K., Trethowan, R., & Pena, R. (2007). Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *Journal of Cereal Science, 46*(3), 293-307.

Pandey, N., Pathak, G. C., & Sharma, C. P. (2006). Zinc is critically required for pollen function and fertilisation in lentil. *Journal of Trace Elements in Medicine and Biology, 20*(2), 89-96.

Peakall, R. O. D., & Peter E. Smouse. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes 6*.1*:* 288-295.

Peakall, Rod, and Peter E. Smouse. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics 28*.19: 2537-2539.

Pozniak, C., Knox, R., Clarke, F., & Clarke, J. (2007). Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. *Theoretical and Applied Genetics*, *114*(3), 525-537.

Prasad, R. (2006). Zinc in Soils and in Plant, Human & Animal Nutrition. *Indian Journal of Fertilisers, 2*(9), 103.

Pritchard, J. K., Stephens, M., Rosenberg, N. A., & Donnelly, P. (2000). Association mapping in structured populations. *The American Journal of Human Genetics, 67*(1), 170-181.

Rafalski, A. (2002). Applications of single nucleotide polymorphisms in crop genetics. *Current Opinion in Plant Biology, 5*(2), 94-100.

Rafalski, J. A. (2010). Association genetics in crop improvement. *Current Opinion in Plant Biology, 13*(2), 174-180.

Raju, P. S., Clark, R. B., Ellis, J. R., & Maranville, J. W. (1990). Effects of species of VA-mycorrhizal fungi on growth and mineral uptake of sorghum at different temperatures. *Plant and Soil, 121*(2), 165-170.

Ramegowda, Y., Venkategowda, R., Jagadish, P., Govind, G., Hanumanthareddy, R. R., Makarla, U., & Guligowda, S. A. (2013). Expression of a rice Zn transporter, OsZIP1, increases [Zn] in tobacco and finger millet transgenic plants. *Plant biotechnology reports, 7*(3), 309-319.

Remington, D. L., Thornsberry, J. M., Matsuoka, Y., Wilson, L. M., Whitt, S. R., Doebley, J., & Buckler, E. S. (2001). Structure of linkage disequilibrium and phenotypic associations in the maize genome. *Proceedings of the National Academy of Sciences, 98*(20), 11479-11484.

Rossi, M., Bitocchi, E., Bellucci, E., Nanni, L., Rau, D., Attene, G., & Papa, R. (2009). Linkage disequilibrium and population structure in wild and domesticated populations of Phaseolus vulgaris L. *Evolutionary Applications*, *2*(4), 504-522.

Sarker, A., El-Askhar, F, Uddin, M. J, Million, E, Yadav, N. K., Dahan, R., and Wolfgang, P. . (2007). Lentil improvement for nutritional security in the developing world. Paper presented at. *ASA- CSSA- SSSA International Annual Meetings, New Orleans, LA,*(4-8 Nov 2007).

Sarker, A., Bayaa, B., & Erskine, W. (2001). Registration of six lentil germplasm lines with resistance to vascular wilt. *Crop Science, 41*(5), 1655-1655.

Sarker, A., Bayaa, B., & Erskine, W. (2005). Combating lentil diseases through host-plant

resistance. Paper presented at the Proceedings of the 1st International Edible Legume Conference in conjuction with the IVth World Cowpea Congress, Durban, South Africa, 17-21 April 2005.

Saskatchewan Agriculture Exports 2015. (2015). *Global Trade Atlas, Statistics Canada (CATSNET).* Retrieved May 16, 2016, from http://www.publications.gov.sk.ca/redirect.cfm?p=74983&i=83881

Shah, D., & Sachdev, H. S. (2001). Effect of gestational zinc deficiency on pregnancy outcomes: summary of observation studies and zinc supplementation trials. *British Journal of Nutrition*, *85*(S2), S101-S108

Sharma, A., Patni, B., Shankhdhar, D., & Shankhdhar, S. (2013). Zinc–an indispensable micronutrient. *Physiology and Molecular Biology of Plants*, *19*(1), 11-20.

Sharpe, A. G., Ramsay, L., Sanderson, L.-A., Fedoruk, M. J., Clarke, W. E., Li, R., . . . Bett, K. E. (2013). Ancient orphan crop joins modern era: gene-based SNP discovery and mapping in lentil. *BMC Genomics*, *14*(1), 192.

Shi, A., Chen, P., Vierling, R., Zheng, C., Li, D., Dong, D., . . . Cervantez, I. (2011). Multiplex single nucleotide polymorphism (SNP) assay for detection of soybean mosaic virus resistance genes in soybean. *Theoretical and Applied Genetics*, *122*(2), 445-457.

Shi, C., Navabi, A., & Yu, K. (2011). Association mapping of common bacterial blight resistance QTL in Ontario bean breeding populations. *BMC Plant Biology, 11*(1), 1.

Shrestha, R., Siddique, K., Turner, D. W., & Turner, N. C. (2009). 12 Breeding and Management to Minimize the Effects of Drought and Improve Water Use Efficiency. *The Lentil*, *100*, 172.

Singh, B., Natesan, S. K. A., Singh, B. K., & Usha, K. (2005). Improving zinc efficiency of cereals under zinc deficiency. *Current Science*, *88*(1), 36-44.

Singh, J. P., Stewart, J., & Karamanos, R. (1987). The zinc fertility of Saskatchewan soils. *Canadian journal of soil science*, *67*(1), 103-116.

Singh, M., Ceccareli, S., & Hambling, J. (1993). Estimation of heritability from varietal trails data. *Theoretical and Applied Genetics 86*: 437-441.

Singh, M., Bisht, I. S., Kumar, S., Dutta, M., Bansal, K. C., Karale, M., ... & Datta, S. K. (2014).

Global Wild Annual Lens Collection: A Potential Resource for Lentil Genetic Base Broadening and Yield Enhancement. *PloS one*, *9*(9), e107781.

Soylak, M., Tuzen, M., Souza, A. S., Korn, M. d. G. A., & Ferreira, S. L. C. (2007). Optimization of microwave assisted digestion procedure for the determination of zinc, copper and nickel in tea samples employing flame atomic absorption spectrometry. *Journal of Hazardous Materials, 149*(2), 264-268.

Stephens, B. W., Cook, D. R., & Grusak, M. A. (2011). Characterization of zinc transport by divalent metal transporters of the ZIP family from the model legume Medicago truncatula. *Biometals*, *24*(1), 51-58.

Streeter, T. C., Rengel, Z., Neate, S. M., & Graham, R. D. (2001). Zinc fertilisation increases tolerance to Rhizoctonia solani (AG 8) in Medicago truncatula. *Plant and Soil, 228*(2), 233-242.

Takkar, P., & Walker, C. D. (1993). The distribution and correction of zinc deficiency *Zinc in Soils and Plants* (pp. 151-165): Springer.

Thavarajah, D., Thavarajah, P., See, C.-T., & Vandenberg, A. (2010). Phytic acid and Fe and Zn concentration in lentil (Lens culinaris L.) seeds is influenced by temperature during seed filling period. *Food Chemistry, 122*(1), 254-259. doi: 10.1016/j.foodchem.2010.02.073

Thavarajah, P., Wejesuriya, A., Rutzke, M., Glahn, R. P., Combs Jr, G. F., & Vandenberg, A. (2011). The potential of lentil (Lens culinaris L.) as a whole food for increased selenium, iron, and zinc intake: preliminary results from a 3 year study. *Euphytica, 180*(1), 123-128.

Tolay, I., Erenoglu, B., Römheld, V., Braun, H. J., & Cakmak, I. (2001). Phytosiderophore release in Aegilops tauschii and Triticum species under zinc and iron deficiencies. *Journal of experimental botany*, *52*(358), 1093-1099.

Tullu, A., Kusmenoglu, I., McPhee, K. E., & Muehlbauer, F. J. (2001). Characterization of core collection of lentil germplasm for phenology, morphology, seed and straw yields. *Genetic Resources and Crop Evolution*, *48*(2), 143-152.

Tullu, A., Banniza, S., Tar'an, B., Warkentin, T., & Vandenberg, A. (2010). Sources of resistance to ascochyta blight in wild species of lentil (Lens culinaris Medik.). *Genetic Resources and Crop Evolution, 57*(7), 1053-1063.

Tullu, A., Diederichsen, A., Suvorova, G., & Vandenberg, A. (2011). Genetic and genomic resources of lentil: status, use and prospects. *Plant Genetic Resources*, *9*(01), 19-29.

Tullu, A., Bett, K., Banniza, S., Vail, S., & Vandenberg, A. (2013). Widening the genetic base of cultivated lentil through hybridization of Lens culinaris 'Eston'and L. ervoides accession IG 72815. *Canadian Journal of Plant Science, 93*(6), 1037-1047.

Ubayasena, L., Bett, K., Tar'an, B., Vijayan, P., & Warkentin. T. (2010). Genetic control and QTL analysis of cotyledon bleaching resistance in green field pea (*Pisum sativum* L.). *Genome 53*: 346–359.

Vandenberg, A., Banniza, S., Warkentin, T., Ife, S., Barlow, B., McHale, S., . . . Bandara, M. (2006). CDC Redberry lentil. *Canadian Journal of Plant Science, 86*(2), 497-498.

Walker, C. F., Ezzati, M., & Black, R. (2009). Global and regional child mortality and burden of disease attributable to zinc deficiency. *European Journal of Clinical Nutrition, 63*(5), 591-597.

Wang, L., Guan, Y., Guan, R., Li, Y., Ma, Y., Dong, Z., ... & Chang, R. (2006). Establishment of Chinese soybean Glycine max core collections with agronomic traits and SSR markers. *Euphytica*, *151*(2), 215-223.

White, P. J., & Broadley, M. R. (2005). Biofortifying crops with essential mineral elements. *Trends in Plant Science, 10*(12), 586-593.

White, P. J., & Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets–iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist, 182*(1), 49-84.

Wong, M. M., Gujaria-Verma, N., Ramsay, L., Yuan, H. Y., Caron, C., Diapari, M., . . . Bett, K. E. (2015). Classification and Characterization of Species within the Genus Lens Using Genotyping-by-Sequencing (GBS). *PloS One, 10*(3), e0122025.

Xia, Y., Li, R., Ning, Z., Bai, G., Siddique, K. H., Yan, G., . . . Guo, P. (2013). Single nucleotide polymorphisms in HSP17. 8 and their association with agronomic traits in barley. *PloS One*, *8*(2), e56816.

Yao, J., Wang, L., Liu, L., Zhao, C., & Zheng, Y. (2009). Association mapping of agronomic traits on chromosome 2A of wheat. *Genetica*, *137*(1), 67-75.

Yu, K., Gu, C. C., Province, M., Xiong, C. J., & Rao, D. C. (2004). Genetic association mapping under founder heterogeneity via weighted haplotype similarity analysis in candidate genes. *Genetic Epidemiology*, *27*(3), 182-191.

Zeng, F., Ali, S., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., & Zhang, G. (2011). The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. *Environmental pollution*, *159*(1), 84-91.

Zhang, Z., Ersoz, E., Lai, C. Q., Todhunter, R. J., Tiwari, H. K., Gore, M. A., ... & Buckler, E. S. (2010). Mixed linear model approach adapted for genome-wide association studies. *Nature Genetics*, *42*(4), 355-360.

APPENDICES

APPENDIX A: ASSOCIATION MAPPING OF ZINC CONCENTRATION IN LENTIL

A.1 Abstract

The knowledge of genetic variation and relationship between genotypes and varieties are important for gaining understanding of genetic variability of different traits. Genetic variability of molecular and physiological markers among lentil genotypes and relationship to important traits like Zn concentration in seed could provide useful potential knowledge for lentil breeding programs that have biofortification as an objective. A lentil association mapping (LAM) panel includes a wide range of genetic diversity among genotypes. Single nucleotide polymorphic (SNP) markers associated with Zn concentration in seeds could be a potential strategy for use in marker-assisted selection and breeding for Zn biofortification of lentil. Genotyping was accomplished using 1150 SNP markers obtained through an Illumina Golden-Gate assay. Association analysis of Zn concentration in lentil seeds identified the two SNP markers significantly associated with Zn concentration in multiple environments and years. Concentration of Zn in lentil seeds was measured for 138 lentil genotypes grown at four environments in 2013 and 2014. Zn concentration in lentil seeds was stable and heritable among studied genotypes. LcC06739p564 and LcC04105p1090 are the two SNP marker associated with Zn concentration in lentil seeds are consistently stable among tested genotypes and environments. These SNP markers are located on chromosomes 3 and 2, respectively, in the draft CDC Redberry lentil genome *v.* 1.2.

A.2 Introduction

The genetic study of the complex quality traits is a potential approach for crop improvement. Phenotypic variation is likely to be significantly associated with the genotype of large germplasm collection. Genotyping a core set of germplasms with contrasting phenotype, tested in different environments provide information about the allelic effects for the trait variation. The genetic basis of seed Zn concentration variation in lentils through association mapping has not been reported to date. The GoldenGate assay from Illumina Inc. (San Diego, CA) was considered to be useful and efficient method for genetic fingerprinting in soybean and common bean (Blair et al., 2013; Hyten et al., 2010). Association mapping provides significant

association among markers and trait of interest. This information could be exploited by lentil breeders to identify specific molecular markers for genetic improvement of lentil. Therefore, the objectives of this study were: 1) to identify SNP markers associated with Zn concentration in lentil seeds, and 2) to assess the genetic diversity and relatedness among the landraces, elite cultivars, and advanced breeding lines.

A.3 Materials and Methods

A.3.1 Plant Material

A previously developed LAM panel of 143 lentil accessions was used for this study. This panel includes popular lentil cultivars, parental genotypes, and breeding lines from the CDC. In addition, 88 landraces obtained from various sources including the International Center for Agricultural Research in the Dry Area (ICARDA) in Aleppo, Syria and the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) in Pullman, Washington was included in this LAM panel. Panel genotypes information was obtained from different resources for example; 'ILL' from http://www.genesys-pgr.org/, 'PI' and 'W6' from http://www.ars-grin.gov/, ICARDA, and http://knowpulse.usask.ca/portal/, and 'CDC' from the Crop Development Centre, University of Saskatchewan. Field evaluations were conducted at two locations in Saskatoon: Sutherland, (STH) and the Saskatchewan Pulse Growers (SPG) farms during the 2013 and 2014 growing seasons. Completely randomized block design (RCBD) was used in each location with six and three replicates in 2013 and 2014, respectively. Around sixty seeds of each genotype were planted in four rows in 1.0m × 1.0m micro-plot with 0.25m distance between the rows.

A.3.2 Phenotyping

Each plot was harvested separately at maturity. Plot samples were threshed and then seed samples were cleaned with distilled water prior to micronutrient analysis. Zn concentration in seed samples was determined by F-AAS. Lentil seed samples were digested and analyzed following the optimized F-AAS procedure described in Chapter 3 for accurate Zn concentration estimation.

A.3.3 Genotyping

All samples were genotyped with the Lc1536 GoldenGate array as described previously (Fedoruk et al., 2013). Genotypic information of these selected genotypes from lentil association

mapping panel was extracted from Khazaei et al. (2016). All genotyping information is available through the KnowPulse web portal (http://knowpulse2.usask.ca/portal/). Genotyped data were clustered for allele calling using GenomeStudio v.2010.3 (Illumina Inc., San Diego, California). Alleles were visually inspected for errors in automatic allele calling and corrected wherever required. Unclear allele calls were reported as missing data.

In association mapping studies, we used SNPs genotyped with at least 70% of the genotypes that showed minor allele frequency (MAF) ≥ 5%. GenAlEx *v.* 6.502. was used to estimate the levels of genetic diversity, polymorphic information content (PIC), and allele frequency within the panel population based on major allele frequency (Peakall and Smouse 2006). The genetic architecture of the 138 genotypes based on 1150 SNP markers was analyzed using STRUCTURE software *v.* 2.3.4. Principle coordinate analysis (PCoA) was performed using GenAlEx *v.* 6.502. output (Peakall and Smouse 2012). The remaining 386 SNP markers were omitted from association analysis due to either having missing data (25 % or higher) or being monomorphic or both.

A.3.4 Statistical Analysis

Statistical analysis was performed by mixed-model analysis of variance (ANOVA) using the SAS *v*. 9.4. In the ANOVA, Zn concentration in lentil seeds was used as the dependent variables. Genotypes and environments were treated as fixed effect whereas locations were random. Equality of variances was performed for each location-year, combined locations, and years by using Levene's test.

A.3.5 Population Structure and Kinship Calculations

Structure software *v.* 2.3.4 was used to determine the relationships within the association panel. It distinguished 138 genotypes into two population sub-groups ($\Delta K = 3$) that corresponded best with two different origins and breeding history of the panel genotypes. Pure and admixture were categorized by their respective genotypic score. Group A included the highest number of genotypes while the rest belonged to group B (Figure A.4). These groups reflect the difference in their origin. Group A includes 57% of total LAM panel. Group A were derived mainly from the elite cultivars and breeding materials developed at the CDC, while the majority of landraces originated from East and West Asia appeared in Group B. However, group B was subdivided in group B1 and B2 for their difference in origin.

A.3.6 Association Analysis

TASSEL *v.* 5 was used to test the marker-trait association between Zn concentration of each genotype and the SNP markers (Bradbury et al., 2007). Twelve hundred forty three unlinked SNP markers distributed throughout the lentil genome were employed to assess the population structure in a collection of 138 genotypes. Rare alleles (alleles frequency < 5%) in the population structure were treated as missing data for controlling false association with the trait.

Both Generalized Linear Model (GLM) and Mixed Linear Model (MLM) were used with TASSEL *v.*5 to calculate the association between markers and traits. MLM method potentially controls over population structure and relatedness within genome-wide association studies. Population structure was considered as fixed effect in the MLM model while the kinship among the individual genotypes was incorporated as the variance-covariance matrix of random effect (Yu et al., 2004; Zhang et al., 2010). Mixed linear model (MLM) was used for the association analysis of the lentil association panel and 1150 SNP markers. Both kinship (K) and Q matrix were used in the MLM association analysis. To observe the interactions of genotype and environment, association analysis was performed for environments individually, for combined environments of the same year, and for all four environments over two year separately. The genetic diversity of the panel genotypes was analysed using Structure and PCoA.

Finally, 1150 SNP markers selected with MAF ≥ 5% were used for association mapping. Significant thresholds were selected to 99.5% of the corresponding eigenvalues in PCoA for the variation for SNP for multiple testing corrections. The relative kinship matrix (K matrix) obtained from the unlinked marker data estimated by TASSEL *v. 5* was combined with the population structure matrix (Q) resulting from the Structure software *v.* 2.3.4 to reduce the spurious false positive rate. Bonferroni correction was used to limit the number of associated markers with lowest p value, leading to high false detection rate (0.05). R^2-marker values were evaluated for significant marker-trait association (at $p < 0.01$).

A.4 Results and Discussion

A.4.1 Phenotypic Data

The total precipitation and mean temperature for two locations at Saskatoon were collected for the growing season (May-August) for both years (2013-14) from the Environment Canada website (http://climate.weather.gc.ca/climate_data/daily_data_e.html?StationID=47707).

The 2014 growing seasons had greater total precipitation (218.9 mm) but a lower mean temperature (15.1^{o}C) compared to 2013 which had mean temperature of 16.2 ^{o}C and a total precipitation of 184.1 mm (Table A.1). Pearson correlation of Zn concentration in lentil seeds was slightly lower (0.5) between locations (SPG-STH) in 2013, lower still (0.4) between locations (SPG-STH) in 2014. However, correlation of seeds Zn concentration was significant (0.6) between years (2013-14) (Table A.3). Seed Zn concentration variability could be due to the difference in micronutrient status in different environments (Table A.2).

A normal frequency distribution was observed for seed Zn concentration of the LAM panel genotypes (Figure A.1-A.3). This reflects the quantitative nature of the trait and environmental interactions affecting Zn concentration in lentil seeds. Table A.3 shows the mean Zn concentration in lentil seeds, and the minimum, maximum, standard deviation, coefficient of variance, and Pearson's correlation coefficients among environments tested in 2013 and 2014 individually and combined over two environments of individual years separately. Analysis of variance revealed a highly significant ($p \leq 0.001$) genotype effect and a significant ($p \leq 0.05$) environment effect on Zn accumulation in lentil seeds (Table A.4). However, significant environment × genotype interaction effects on seed Zn concentration were not observed in four environments in 2013-14. The C.V. for seed Zn concentration among all environments was 12.6 % (Table A.4). Variance components were used to calculate the heritability of Zn concentration in lentil seeds by considering both locations of each year separately.

Table A.1. Mean temperature (^{o}C) and total precipitation (mm) during the lentil growing season (May-August) in 2013-14 at Saskatoon.

	May		June		July		Aug	
Climate data	2013	2014	2013	2014	2013	2014	2013	2014
Mean temperature (^{o}C)	13.0	10.1	15.5	14.1	17.4	18.3	18.9	17.9
Total precipitation (mm)	15.9	61.1	117.7	94.8	35.6	44.5	14.9	18.5

Table A.2. Soil analysis from two field locations at Saskatoon (Sutherland farm (STH) and Saskatchewan pulse growers farm (SPG)) in 2014.

Location	Depth (inches)	Texture	pH	Salinity Rating	Organic Matter (%)	DTPA-extractable [Zn] (mg kg^{-1})
STH	0-6	Clay Loam	6.6	NS	3.6	1.0
SPG	0-6	Loam	7.6	NS	4.0	1.5

Note: ***NS***, Non-saline

Table A.3. Zn concentration variation in lentil seeds from Saskatchewan pulse grower (SPG) and Sutherland (STH) farms in 2013-14.

	[Zn] (mg kg^{-1}) in 2013		[Zn] (mg kg^{-1}) in 2014		Average [Zn] (mg kg^{-1})	
Environment	SPG	STH	SPG	STH	2013	2014
Maximum	51.65	47.07	53.60	50.67	48.13	50.70
Minimum	25.18	27.50	23.46	26.71	27.14	27.69
Average	38.86	35.70	37.87	38.61	37.28	38.24
Correlation	0.53		0.40		0.64	
STDEV.P	5.45	2.87	5.31	4.26	3.69	4.00
C.V. (%)	14.02	8.05	14.01	11.03	9.89	10.47

Note: ***[Zn]***, Zn concentration, measured in seeds by F-AAS (mg kg^{-1})

Table A.4. F-values from analysis of variance (ANOVA) and Coefficient of variance (C.V.) of Zn concentration in lentil seeds of association panet grown at the Saskatchewan Pulse Growers and Sutherland Agricultural Farm near Saskatoon in 2013 and 2014.

Effect	df	F-value
Environment	3	8.90^{*}
Genotype	137	3.40^{**}
Environment × Genotype	411	0.75^{ns}
C.V. (%)	12.60	

Note: ns, not significant; *, significant at $p \leq 0.01$, and **, significant at $p \leq 0.001$.

A.4.2 Estimation of Variance Components and Heritability

The heritability of Zn concentration in lentil seeds was stable in both environments in both 2013 and 2014 (Table A.5). Highly heritable seed Zn concentration was observed in 2014 and 2015 (H^2 = 0.61 and 0.56, respectively). Environmental variation of seed Zn concentration in lentil seeds reflects the broad-sense heritability of the trait in different environments.

Table A.5. Estimates of variance components and broad-sense heritability of Zn concentration in lentil seeds for the association mapping panel grown at the Saskatchewan Pulse Growers and Sutherland Agricultural farm, Saskatoon in 2013 and 2014.

Variance component	2013	2014
σ^2 Genotype	8.31	9.01
σ^2 Genotype × Environment	9.81	13.36
σ^2 Environment	0.98	0.95
σ^2 Phenotype	13.71	16.17
H^2	0.61	0.56

Note: $\boldsymbol{H^2}$,broad-sense heritibility of Zn concentration in lentil seeds

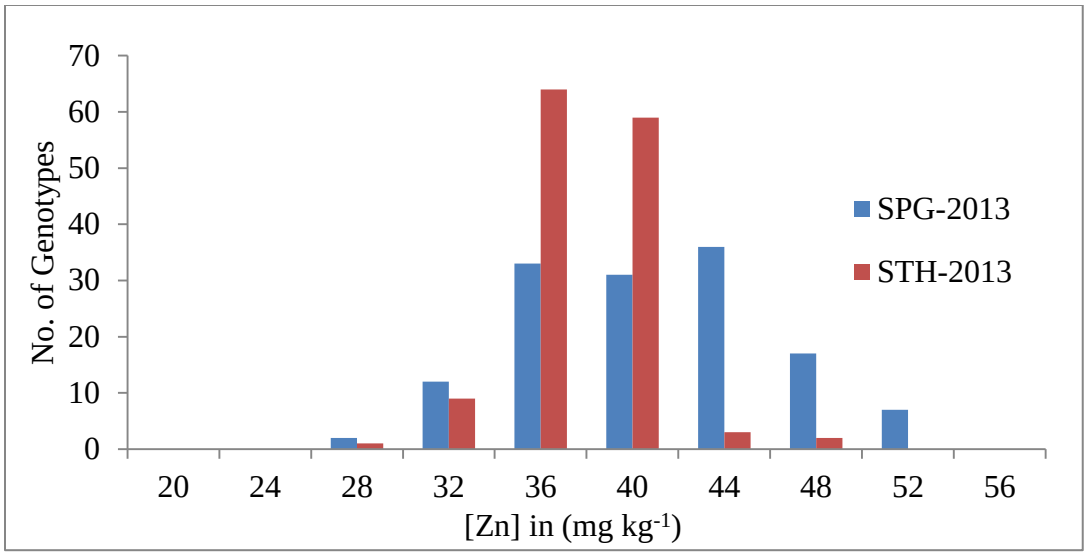

Figure A.1. Frequency distribution of mean seed Zn concentration in 2013 for 138 genotypes the from lentil association mapping (LAM) panel grown at the Saskatchewan Pulse Growers (SPG): Mean of 138 LAM genotypes = 38.86; Sutherland (STH) farm: Mean of 138 LAM genotypes = 35.70.

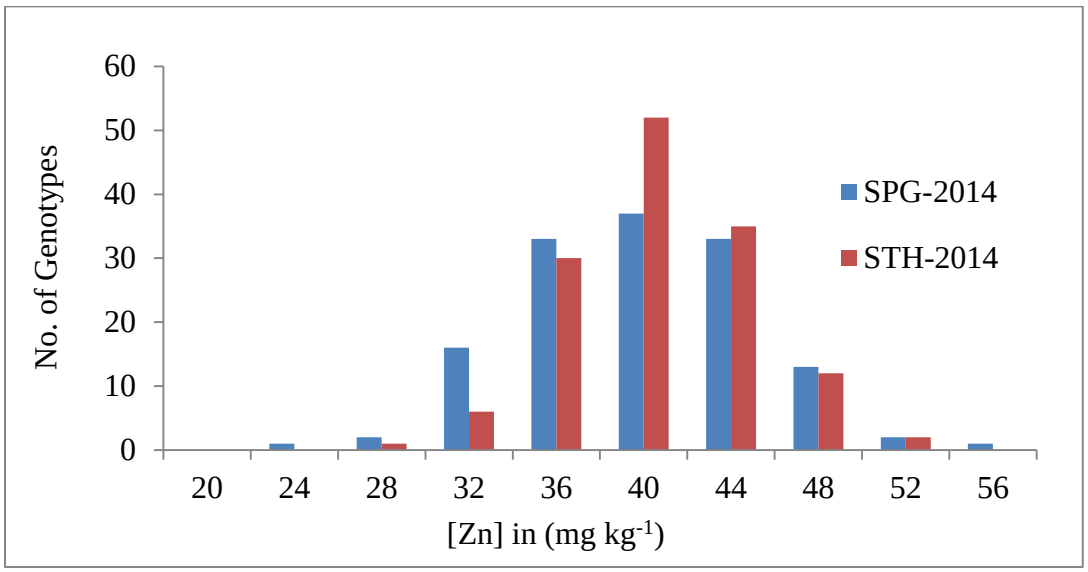

Figure A.2. Frequency distribution of mean seed Zn concentration for 138 genotypes of the lentil association mapping (LAM) panel grown in 2014 at the Saskatchewan Pulse Growers (SPG): Mean of 138 LAM genotypes = 37.87; Sutherland (STH) farm: Mean of 138 LAM genotypes = 38.61.

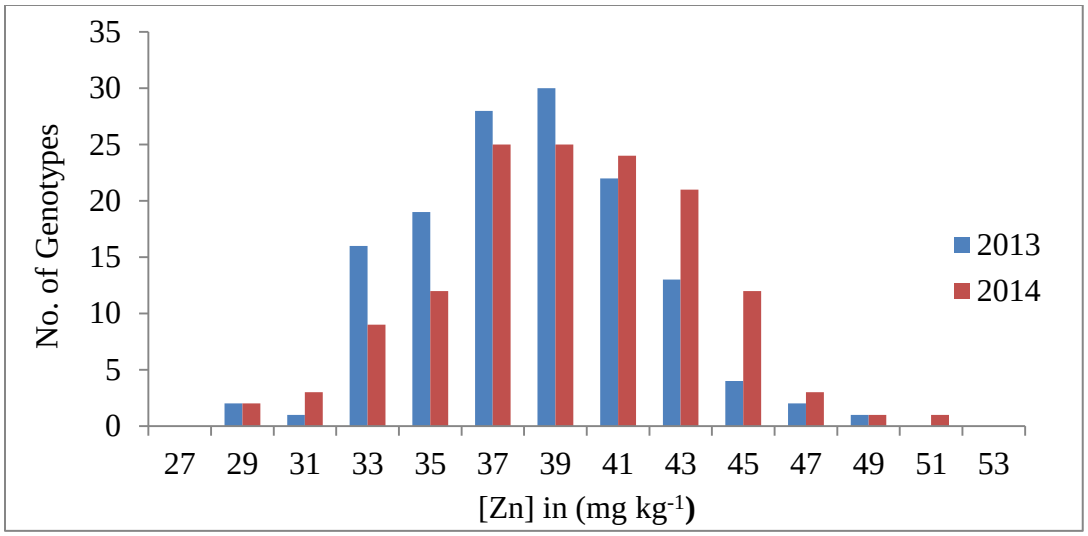

Figure A.3. Frequency distribution of seed Zn concentration of 138 genotypes in the lentil association mapping (LAM) panel of 2013 and 2014 grown at the Saskatchewan Pulse Growers and Sutherland farm.

The phylogenetic tree was constructed from Tassel *v.* 4.0 UPGMA and the resulting tree was visualized using Interactive Tree of Life (Itol) v. 3.0 (Letunic & Bork, 2011). This dendrogram showed two major ancestor groups (A and B) for the lentil association mapping panel genotypes. Dendrogram tree branch Group B immediately subdivided into two braches designated as Group B1 and Group B2. It demonstrates the close history of ancestry origin of both Groups B1 and B2 (Figure A.4). Grouping among the 138 panel genotypes revealed the clustering based on geographical origin and available SNP markers information. Genetic diversity was also observed for landraces, breeding lines, and lentil cultivars. Selection of divergent parental genotypes for breeding should be made considering the genetic distance rather than geographical distance.

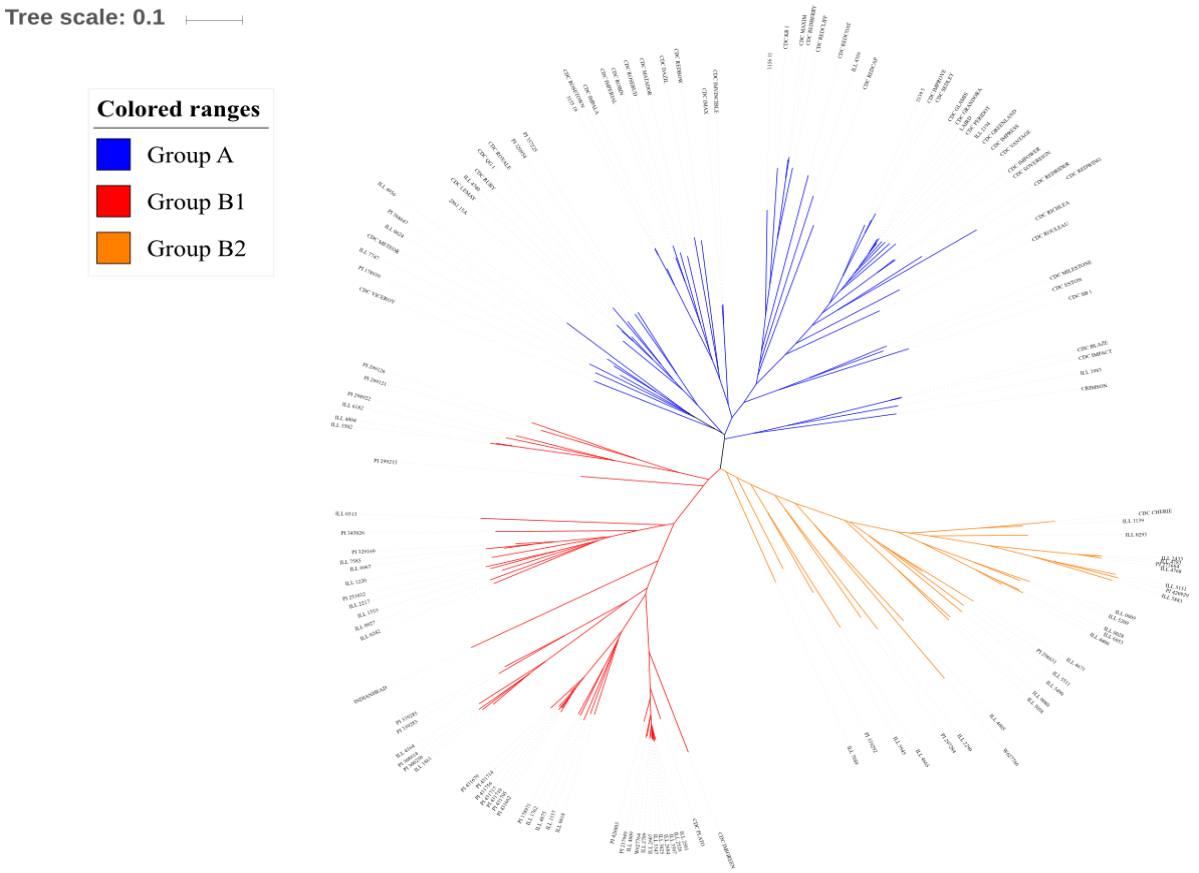

Figure A.4. Unrooted phylogenetic tree of 138 lentil genotypes from the lentil association mapping panel based on 1150 SNP markers.

A.4.3 Association Analysis

Ten SNP markers reported (Table A.6) were significantly associated with seed Zn concentration in both years. Both cumulative *p*-value and false detection rate (FDR at 0.05%) were taken into consideration for ranking the SNP markers. The top four SNP markers were associated with seed Zn concentration in multiple environments. The markers LcC06739p564 and LcC04105p1090 were significantly associated in both years. Both markers are found on chromosomes 3 and 2, respectively in the draft 'CDC Redberry' lentil genome *v.* 1.2. The percentage identities of these SNP markers are 97.52 and 100, respectively with the position in draft lentil genome assembly.

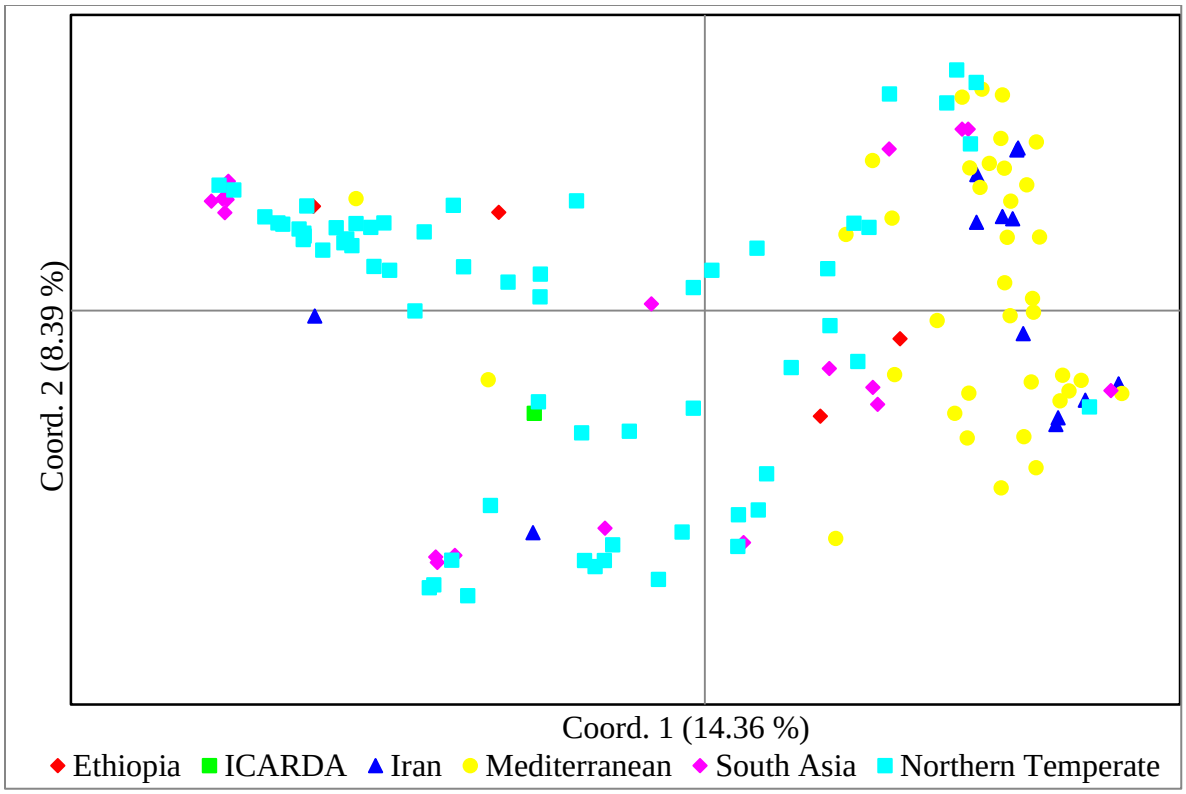

Figure A.5. Principal Coordinate analysis (PCoA) of the138 lentil genotypes using 1150 SNP markers. Each colored symbol represents the place of origin.

Globally lentils are grown in three major agro-ecological zones with different temperature and photoperiod regimes during growing season (Tullu et al., 2011). South Asia, Mediterranean, and northern temperate are the three major lentil growing zones in the world. Mapping panel genotypes were grouped into three main groups considering the origin, breeding history, and pedigree of the genotypes.

Table A.6. Significant markers associated with Zn concentration in lentil seeds estimated with both Multiple Linear Model (MLM) and Principal Component Analysis (PCA+K) and Multiple Linear Model (MLM) and (Q+K) using 1150 SNP markers against 138 diverse lentil genotypes.

Marker	Chromosome Number	Position	MLM (PCA+K) Model				MLM (Q+K) Model			
			F value	*p* value	FDR (0.05)	R^2	F value	*p* value	FDR (0.05)	R^2
LcC06739p564	3	115106518	15.2	0.0002	4.3E-05	12.0	16.0	0.0001	4.35E-05	12.4
LcC04105p1090	2	309436420	11.5	0.0010	2.2 E-04	10.5	11.7	0.0009	2.2 E-04	10.5
LcC03907p461	1	71589381	8.5	0.0004	8.7E-05	14.0	9.0	0.0002	8.70E-05	14.6
LcC18132p1029	3	103760965	6.7	0.0018	3.0 E-04	10.3	6.6	0.0019	3.9 E-04	10.1
LcC01084p238	1	71650376	7.9	0.0006	1.3 E-04	12.5	7.7	0.0007	1.7 E-04	12.0
LcC05435p444	ND*	33216	6.4	0.0023	4.4 E-04	10.2	-	-	-	-
LcC01183p864	4	239152233	6.6	0.0018	3.9 E-04	9.9	6.6	0.0018	3.5 E-04	9.8
LcC09698p304	2	1316987	6.8	0.0016	2.6 E-04	10.9	6.8	0.0016	3.0 E-04	10.8
LcC08037p136	4	229287826	7.7	0.0007	1.7 E-04	7.7	8.0	0.0005	1.3 E-04	11.9
LcC11556p306	ND	ND	6.7	0.0018	3.5 E-04	11.7	7.0	0.0013	2.6 E-04	12.1

Note: ***ND***, LcContig216832

ND, not determined

The lentil genotypes used in this study were obtained from successful varieties, crossing material and landraces originating from different geographical regions, and diversity in the lentil genome was expected to have higher resolution. The broad-sense heritability for seed Zn concentration was estimated at 0.61 and 0.56 in 2013 and 2014, respectively (Table A.5). This indicates the potential use of molecular markers in marker-assisted lentil breeding for increasing seed Zn concentration. Consistent SNP markers associated with seed Zn concentration for all four tested environments showed the influence of both genotype and genotype × environment interaction on Zn accumulation. Significant interactions between environments (location and year) and significant differences among the genotypes at all environments were observed for seed Zn concentration. SNP markers were detected using the both genetic and phenotypic information (Zn concentration) at both locations in 2013-14.

LAM population was categorized into six main groups based on their source of origin; 1) Ethiopia, 2) ICARDA, 3) Iran, 4) Mediterranean, 5) South Asia, and 6) Northern Temperate. These are the main climatic regions of lentil production across the world. About 50% of total world lentil production is from two major lentil production regions such as northern temperate (Canada) and Mediterranean (FAO, 2015). Genetic structure of the LAM population was analyzed by principal coordinate analysis (PCoA) using GenAlex (Figure A.5). It revealed the molecular variance among and within three assign groups. Results from STRUCTURE and UPGMA cluster analysis and PCoA were highly consistent. Dendrogram tree analysis using Interactive Tree of Life (Itol) (Figure A.4) also revealed three groups of genotypes (group A, B1, and B2) in the studied LAM population. Group B1 and B2 are closely related than group A. In this current study, three major groups of genotypes reflecting the origin, evolution, and their pedigree breeding history are identified using relatively higher density of SNIP markers and selected genotypes for LAM population. This study suggested the presence of putative genes that controls the Zn accumulation in lentil seeds on chromosome 3, 2, and 1. The R^2 value of the SNP markers were 10.5, 12.4, and 14.6 (%) for LcC04105p1090, LcC06739p564, and LcC03907p461, respectively, from MLM (Q + K) maker-trait association analysis. These three markers had R^2 value of 10.5, 12.0, and 14.0, respectively, from MLM (PCA) analysis (Table A.6). These R^2 value for the reported markers indicated the fraction of total Zn concentration variation in lentil seeds explained by the method of analysis.

APPENDIX B: GENOTYPE × ENVIRONMENT INTERACTION ON ZN ACCUMULATION IN SEEDS OF LENTIL INTERSPECIFIC RECOMBINANT INBRED POPULATION (LR-26)

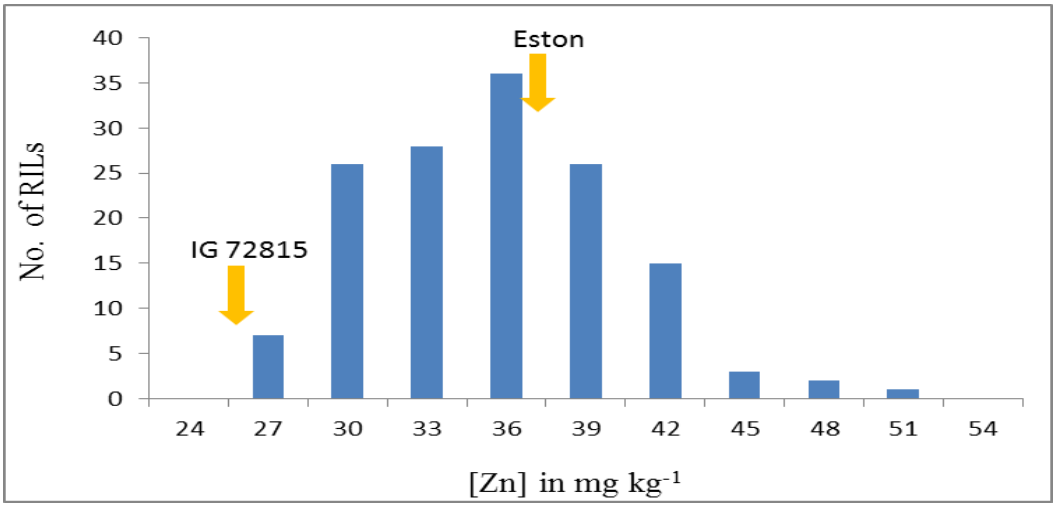

Figure B.1. Frequency distribution of 142 recombinant inbred lines (RILs) derived from IG 72815/Eston (LR-26) for their [Zn] (mg kg^{-1}) based on the means of 2014. Crop Science Field Laboratory (CSFL): Mean of RILs = 34.1.

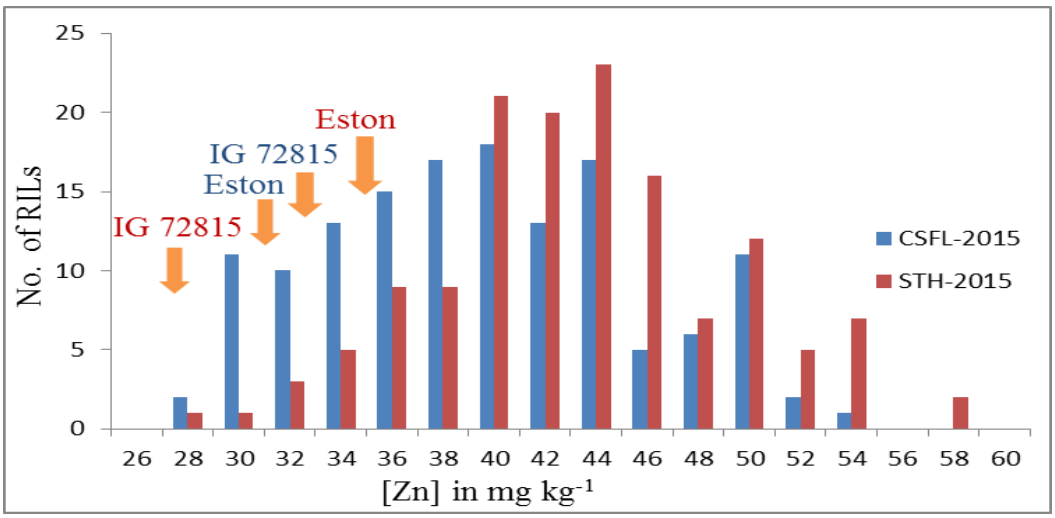

Figure B.2. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 139 interspecific lentil recombinant inbred population (RILs) derived from IG 72815/Eston (LR-26) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 38.5; Sutherland (STH) farm: Mean of RILs = 42.3.

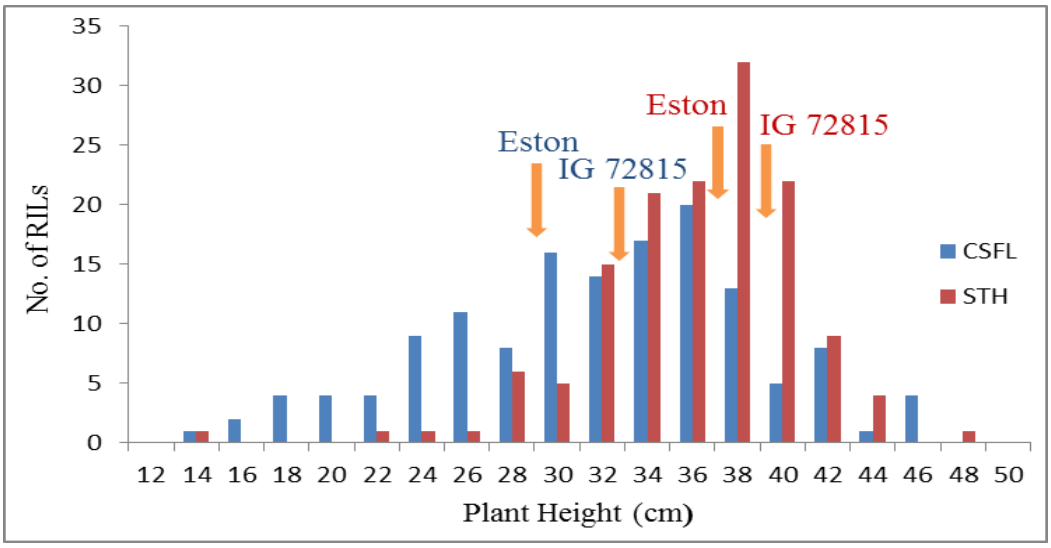

Figure B.3. Frequency distribution of plant height (cm) of 139 interspecific lentil recombinant inbred population (RILs) derived from IG 72815/Eston (LR-26) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 31.09; Sutherland (STH) farm: Mean of RILs = 35.26.

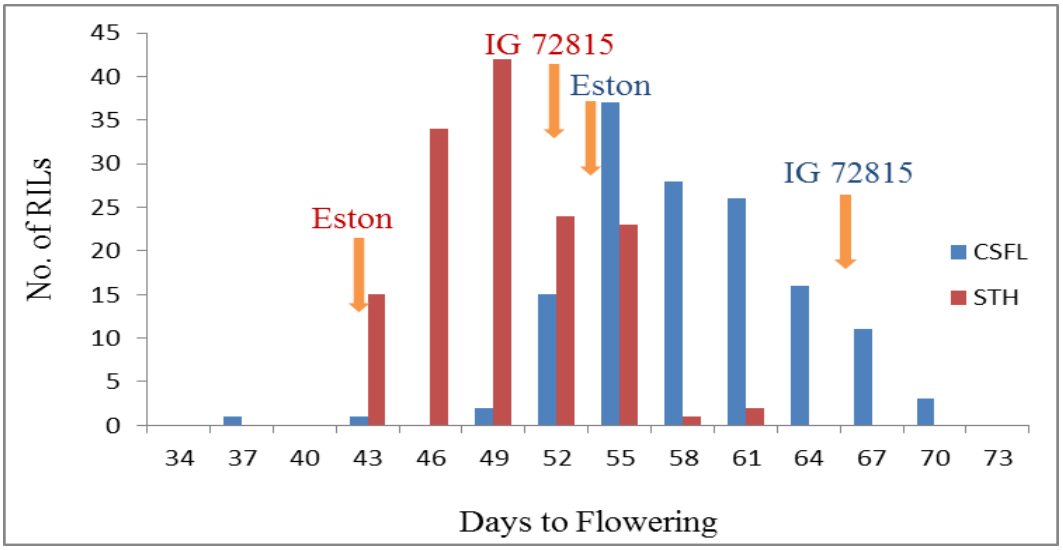

Figure B.4. Frequency distribution of days to flowering of 139 interspecific lentil recombinant inbred population (RILs) derived from IG 72815/Eston (LR-26) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 55.92; Sutherland (STH) farm: Mean of RILs = 47.28.

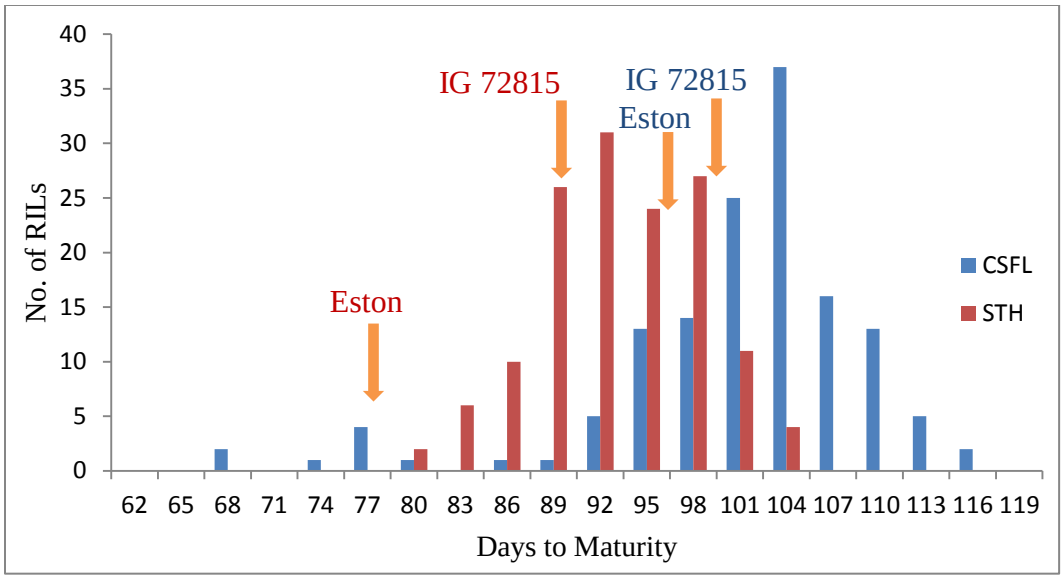

Figure B.5. Frequency distribution of days to maturity of 139 interspecific lentil recombinant inbred population (RILs) derived from IG 72815/Eston (LR-26) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 97.37; Sutherland (STH) farm: Mean of RILs = 90.85.

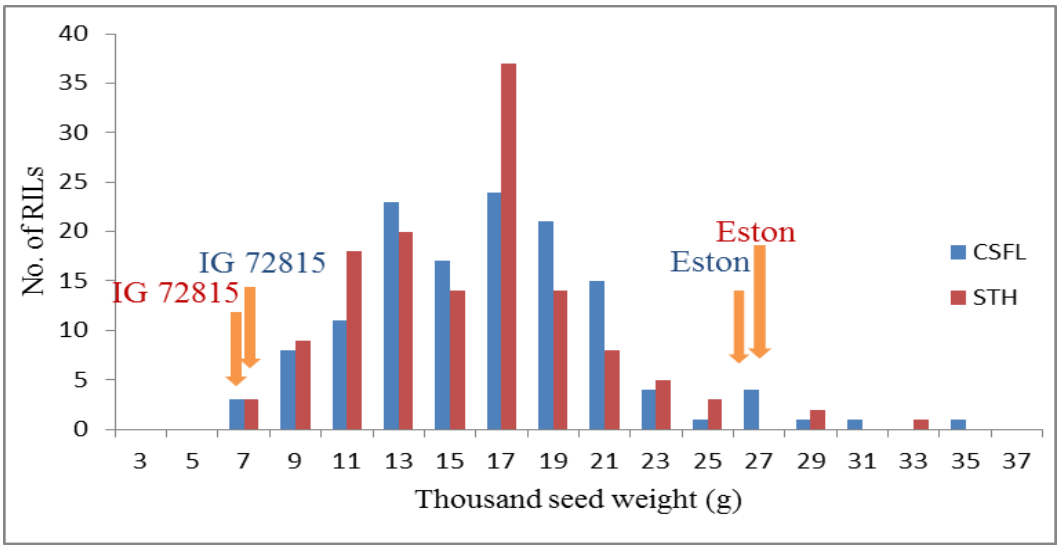

Figure B.6. Frequency distribution of thousand seed weight (g) of 139 interspecific lentil recombinant inbred population (RILs) derived from IG 72815/Eston (LR-26) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 16.38; Sutherland (STH) farm: Mean of RILs = 15.58.

Table B.1. Mean, standard deviation (Std. Dev.), minimum, and maximum of five traits (average of plant height, days to flowering, days to maturity, 1000 seed weight, and seed Zn concentration) of 139 RILs of LR-26 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015.

Parents\Trait	PH	DTF	DTM	TSW	[ZN]
Eston	33.5	48.0	87.3	27.1	32.8
IG 72815	31.2	58.8	94.7	5.7	30.5
Minimum	18.2	36.0	76.3	7.1	29.7
Maximum	42.3	62.7	106.3	32.5	52.5
Average	33.2	52.5	95.9	15.1	40.6
Std. Dev.	4.5	3.8	5.6	4.3	4.8

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}).

Table B.2. Pearson correlation coefficients for five measured traits (average of plant height, days to flowering, days to Maturity, 1000 seed weight, and seed Zn concentration) of 139 RILs of LR-26 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015.

	PH	DTF	DTM	TSW	[ZN]
PH	1	-0.15^{ns}	-0.08^{ns}	0.23^{**}	0.13^{ns}
DTF		1	0.55^{***}	-0.39^{***}	0.09^{ns}
DTM			1	-0.40^{***}	0.20^{*}
TSW				1	-0.25^{**}
[ZN]					1

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}); ns, not significant; *, significant at $p \leq 0.05$; **, significant at $p \leq 0.01$; ***, indicates significant correlation at $p \leq 0.001$.

Table B.3. Summary of analysis of variance for thousand seed weight and zinc concentration for 166 lentil recombinant inbred lines of LR-26 derived from a cross between IG 72815/Eston evaluated at Sutherland farms and Crop Science Field Laboratory in 2015.

Effect	df	[Zn]	TSW
Environment	1	13.29^{*}	3.42^{ns}
Genotype	165	2.89^{**}	6.02^{**}
Environment × Genotype	157	1.53^{**}	1.59^{**}

Note: ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}).
ns, not significant; *, significant at $p \leq 0.05$, and **, significant at $p \leq 0.001$.

Table B.4. Estimates of variance components and broad-sense heritability of thousand seed weight and zinc concentration in 167 lentil recombinant inbred line population (LR-26) evaluated at Crop Science Field Laboratory and Sutherland farm in 2015.

Variance component	[Zn]	TSW
σ^2 Genotype	3.73	6.40
σ^2 Genotype × Environment	3.07	2.92
σ^2 Environment	16.0	15.46
σ^2 Phenotype	7.94	10.44
H^2	0.47	0.61

Note: ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1});
$\boldsymbol{H^2}$,broad-sense heritibility.

APPENDIX C: GENOTYPE × ENVIRONMENT INTERACTION EFFECTS ON ZN ACCUMULATION IN SEEDS OF LENTIL INTERSPECIFIC RECOMBINANT INBRED POPULATION (LR-59)

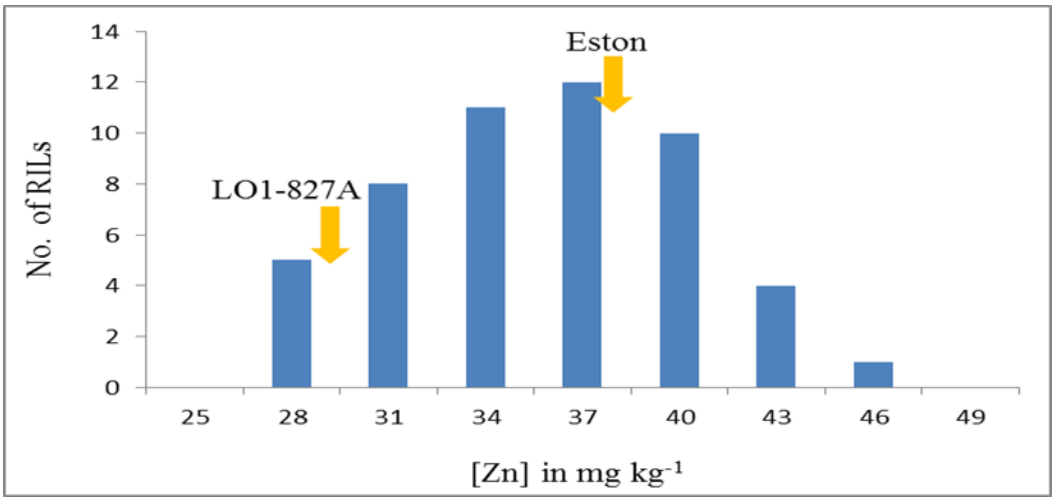

Figure C.1. Frequency distribution of 49 recombinant inbred lines (RILs) derived from L01-827A/Eston (LR-59) for their [Zn] (mg kg^{-1}) based on the means of 2014. Crop Science Field Laboratory (CSFL): Mean of RILs = 34.2.

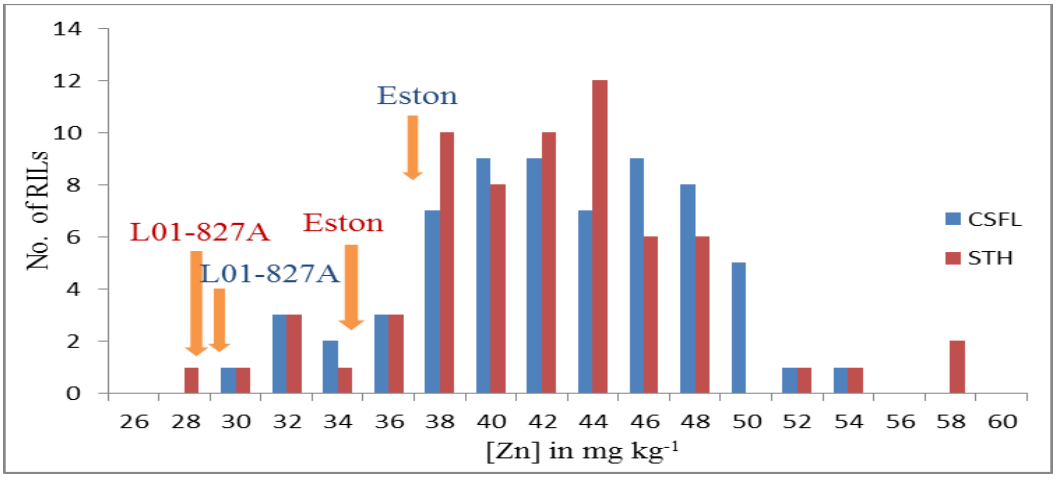

Figure C.2. Frequency distribution of seed zinc concentration (mg kg^{-1}) of 63 interspecific lentil recombinant inbred population (RILs) derived from L01-827A/Eston (LR-59) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 41.7; Sutherland (STH) farm: Mean of RILs = 39.9.

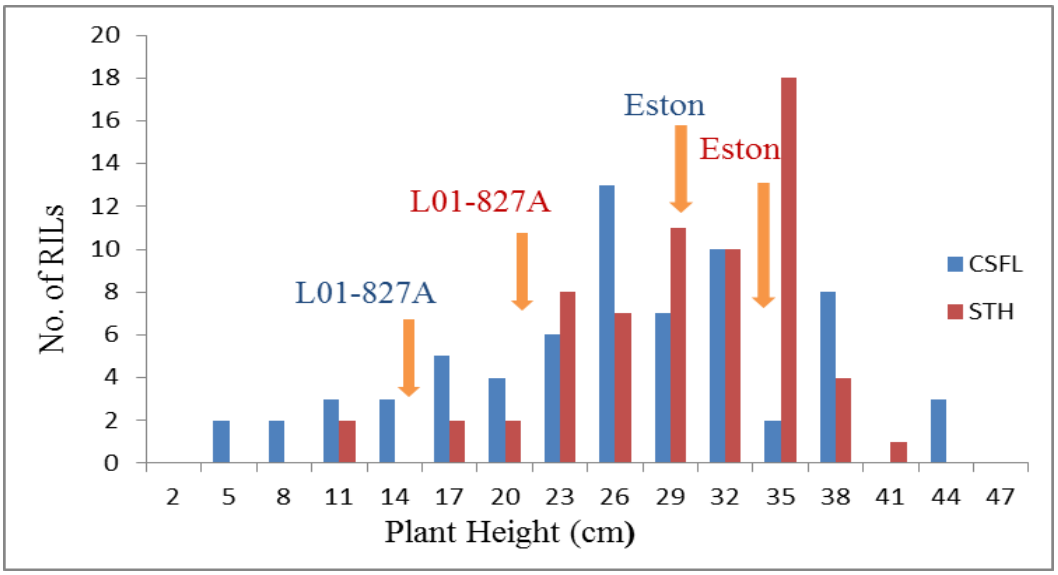

Figure C.3. Frequency distribution of seed plant height (cm) of 63 interspecific lentil recombinant inbred population (RILs) derived from L01-827A/Eston (LR-59) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 24.80; Sutherland (STH) farm: Mean of RILs = 28.28.

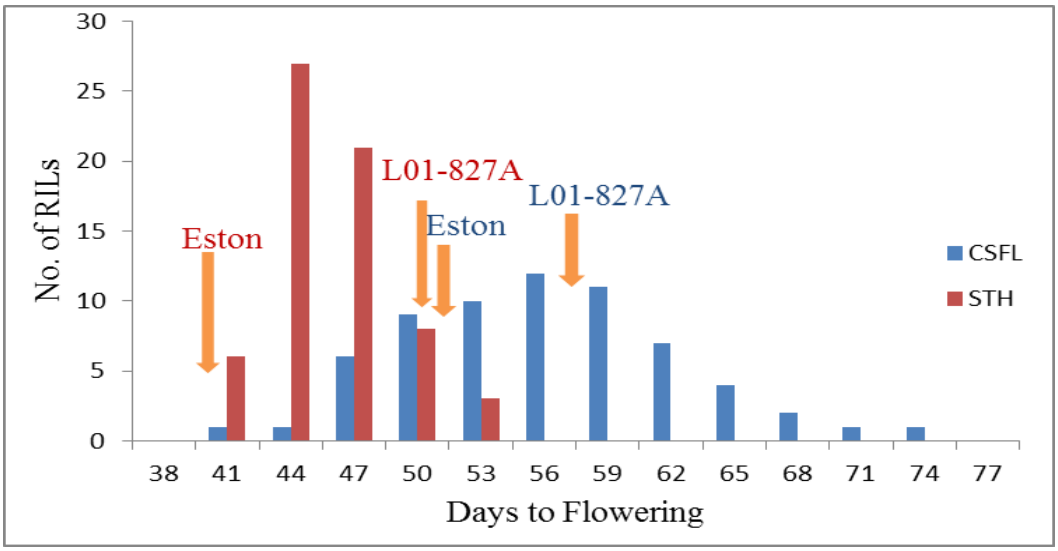

Figure C.4. Frequency distribution of days to flowering of 63 interspecific lentil recombinant inbred population (RILs) derived from L01-827A/Eston (LR-59) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 54.75; Sutherland (STH) farm: Mean of RILs = 44.46.

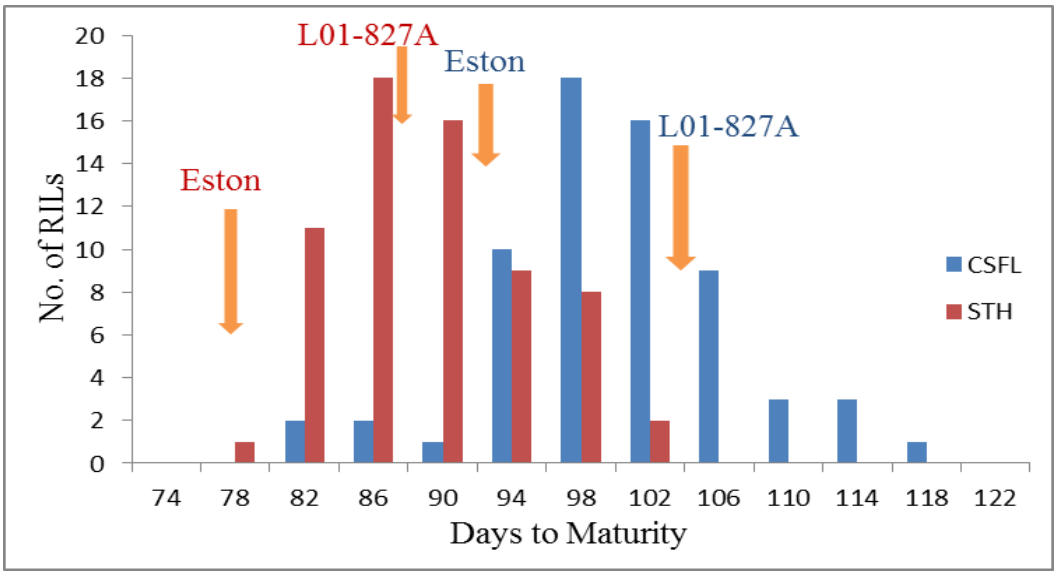

Figure C.5. Frequency distribution of days to maturity of 63 interspecific lentil recombinant inbred population (RILs) derived from L01-827A/Eston (LR-59) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 98.72; Sutherland (STH) farm: Mean of RILs = 87.67.

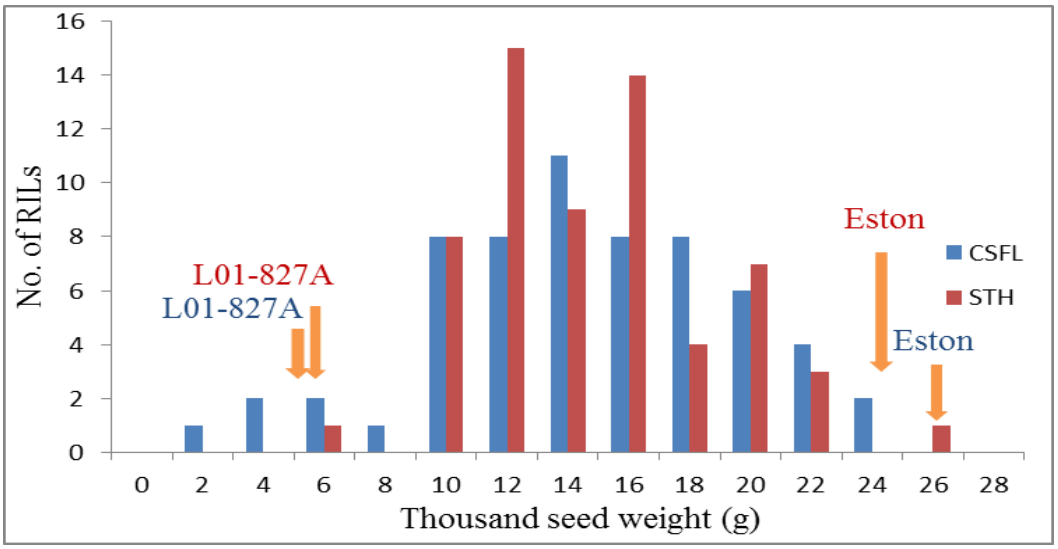

Figure C.6. Frequency distribution of thousand seed weight (g) of 63 interspecific lentil recombinant inbred population (RILs) derived from L01-827A/Eston (LR-59) were grown in 2015. Crop Science Field Laboratory (CSFL): Mean of RILs = 13.58; Sutherland (STH) farm: Mean of RILs =13.84.

Table C.1. Mean, standard deviation (Std. Dev.), minimum, and maximum of five traits (average of plant height, days to flowering, days to Maturity, 1000 seed weight, and seed Zn concentration) of 63 RILs of LR-59 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015.

Parents\Trait	PH	DTF	DTM	TSW	[ZN]
Eston	32.0	46.0	84.8	25.3	35.7
L1-02-827A	18.7	54.0	95.3	5.5	29.0
Minimum	7.0	40.5	82.8	5.3	30.6
Maximum	39.0	59.8	104.2	21.5	53.2
Average	26.6	49.6	93.3	13.8	41.6
Std. Dev.	7.1	3.9	4.7	3.9	4.7

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}).

Table C.2. Pearson correlation coefficients for average plant height, days to flowering, days to Maturity, 1000 seed weight, and seed Zn concentration for 63 RILs of LR-59 population grown at two environments (Crop Science Field Laboratory and Sutherland farm) in 2015.

	PH	DTF	DTM	TSW	[ZN]
PH	1	-0.22^{ns}	-0.25^{*}	0.49^{***}	0.00^{ns}
DTF		1	0.40748^{***}	-0.44561^{***}	0.06712^{ns}
DTM			1	-0.37444^{**}	0.15493^{ns}
TSW				1	-0.28833^{*}
[ZN]					1

Note: ***PH***, plant height (cm); ***DTF***, days to flowering; ***DTM***, days to maturity; ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}); ns, not significant ; *, significant at $p \leq 0.05$; **, significant at $p \leq 0.01$; ***, indicates significant correlation at $p \leq 0.001$.

Table C.3. Summary of analysis of variance for thousand seed weight and zinc concentration for 66 lentil recombinant inbred lines of LR-59 derived from a cross between L01-827A/Eston evaluated at Sutherland farms and Crop Science Field Laboratory in 2015.

Effect	df	[Zn]	TSW
Environment	1	0.10^{ns}	0.57^{ns}
Genotype	65	4.38^{**}	1.13^{ns}
Environment × Genotype	65	1.53^{*}	0.85^{ns}

Note: ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1}).
ns, not significant; *, significant at $p \leq 0.05$, and **, significant at $p \leq 0.001$.

Table C.4. Estimates of variance components and broad-sense heritability of thousand seed weight and zinc concentration in 66 lentil RILs population (LR-59) evaluated at Crop Science Field Laboratory and Sutherland farm in 2015.

Variance component	[Zn]	TSW
σ^2 Genotype	3.42	0.81
σ^2 Genotype × Environment	1.87	0.55
σ^2 Environment	10.20	10.32
σ^2 Phenotype	6.06	2.64
H^2	0.56	0.31

Note: ***TSW***, 1000 seed weight (g); ***[Zn]***, Zn concentration (mg kg^{-1});
$\boldsymbol{H^2}$, broad-sense heritibility.

APPENDIX D: SEED ZN CONCENTRATIO AT THREE HARVESTS OF 58 WILD LENTIL GENOTYPES IN 2014

Lens species	Genotype	[Zn] in1st harvest (mg kg^{-1})	[Zn] in 2nd harvest (mg kg^{-1})	[Zn] in 3rd harvest (mg kg^{-1})
Lens orientalis	IG 72611	28.8	36.5	35.8
Lens orientalis	IG 72643	40.1	33.1	38.3
Lens orientalis	IG 141573	36.2	36.8	37.5
Lens orientalis	IG 144228	34.3	No harvest	No harvest
Lens orientalis	IG 136777	37.6	37.9	36.7
Lens orientalis	IG 136802	31.8	31.0	26.6
Lens orientalis	IG 136814	28.1	32.1	29.7
Lens orientalis	IG 136815	33.8	31.6	30.6
Lens orientalis	IG 136819	30.6	29.3	No harvest
Lens orientalis	IG 137358	32.4	28.7	31.6
Lens orientalis	IG 137365	36.4	33.2	33.5
Lens orientalis	IG 137423	44.0	40.6	27.9
Lens orientalis	IG 137429	48.9	41.8	33.8
Lens orientalis	IG 137552	44.5	39.7	39.7
Lens orientalis	IG 137606	27.0	26.5	31.8
Lens orientalis	IG 140379	36.2	33.4	40.4
Lens orientalis	IG 140389	35.2	27.8	26.1
Lens orientalis	IG 140891	34.1	27.9	28.6
Lens orientalis	IG 140893	35.5	25.7	26.8
Lens orientalis	IG 140968	41.5	39.6	39.9
Lens orientalis	IG 140970	36.6	34.4	35.4
Lens orientalis	IG 140972	36.4	30.2	31.3
Lens tomentosus	PI 572390	30.6	45.5	42.4
Lens tomentosus	IG 72613	34.5	39.5	46.1
Lens tomentosus	IG 72534	36.3	32.9	36.5
Lens tomentosus	IG 72614	46.3	41.8	34.5
Lens tomentosus	IG 72616	40.4	32.2	41.3
Lens tomentosus	IG 72672	39.4	38.6	39.1
Lens tomentosus	IG 72805	34.3	37.8	38.4
Lens tomentosus	IG 72830	33.9	26.4	30.3
Lens tomentosus	IG 72831	33.0	29.1	33.5
Lens lamottei	IG 110810	51.2	34.6	36.4
Lens lamottei	IG 110813	38.8	38.3	39.7

Lens species	Genotype	[Zn] in1st harvest (mg kg-1)	[Zn] in 2nd harvest (mg kg^{-1})	[Zn] in 3rd harvest (mg kg^{-1})
Lens odemensis	IG 72623	39.5	35.2	33.3
Lens odemensis	IG 136788	25.0	22.0	24.0
Lens odemensis	IG 136800	37.4	No harvest	No harvest
Lens odemensis	IG 136662	29.1	26.0	No harvest
Lens odemensis	IG 139285	32.9	34.1	25.9
Lens odemensis	IG 72845	24.3	24.1	22.5
Lens ervoides	IG 72815	24.3	24.1	32.9
Lens ervoides	IG 140884	27.5	21.5	30.5
Lens ervoides	IG 141656	38.3	31.7	26.9
Lens ervoides	IG 140910	37.3	36.2	25.8
Lens ervoides	IG 140927	34.5	29.0	25.4
Lens ervoides	IG 140929	31.6	28.2	18.7
Lens ervoides	IG 140935	43.5	33.3	27.1
Lens nigricans	IG 116024	37.4	33.3	29.1
Lens nigricans	IG 136681	35.5	40.5	39.9
Lens nigricans	IG 136637	30.4	29.7	35.4
Lens nigricans	IG 136647	28.3	27.5	19.7
Lens nigricans	IG 136649	29.8	29.9	34.5
Lens nigricans	IG 136650	33.8	32.9	33.7
Lens nigricans	IG 136651	30.5	25.3	22.9
Lens nigricans	IG 136682	47.6	43.1	41.2

APPENDIX E: SEED ZN CONCENTRATION AND NODULATION DATA FROM A SUB-SET OF LR-26 AT SASKATOON IN 2014

Genotype	Days to flower	Plant height at flowering (cm)	Nodulation	Seed yield (g)	Seed weight (g)	[Zn] in seeds (mg kg^{-1})
Eston	45	38.2	2(14)	254.4	33.6	29.4
IG 72815	55	24.0	3(67)	83.0	7.4	27.1
LR 26-19	48	24.8	2(13)	171.9	20.6	33.5
LR 26-41	52	27.2	3(28)	92.2	16.5	40.6
LR 26-79	50	25.2	2	20.8	9.8	40.0
LR 26-98	54	25.4	2	69.0	17.5	37.8
LR 26-110	60	19.4	3	29.2	10.5	40.5
LR 26-128	50	25.0	2	39.1	10.8	36.8
LR 26-145	53	28.0	2	117.1	17.4	34.5
LR 26-165	51	29.2	1	99.2	13.6	37.9
LR 26-184	50	31.4	2	172.1	26.4	29.2
LR 26-216	52	32.8	1	153.5	21.2	32.0
LR 26-241	55	37.8	3 (145)	253.8	27.1	32.2
LR 26-253	52	31.6	3 (56)	89.7	22.5	44.2
LR 26-274	55	35.0	3 (62)	135.7	22.1	32.6
LR 26-288	55	37.2	3 (44)	186.2	22.1	35.4
LR 26-297	49	31.0	3 (122)	123.9	19.8	34.6
LR 26-300	51	37.8	1	158.5	19.9	40.1

Note: Nodulation data was taken from single plots; ICARDA nodulation descriptor scale was followed (Nodule number: None =0, Less than 10 = 1, 11-20 = 2, More than 20 = 3)

*Seeded on May 30, 2014

*Because of poor seed germination, 2015 field trail was abandoned.

APPENDIX F: SEED ZN CONCENTRATION AND NODULATION DATA FROM A SUB-SET OF LR-59 AT SASKATOON IN 2014

Genotype	Days to flower	Plant height at flowering (cm)	Nodulation	Seed yield (g)	Seed weight (g)	[Zn] in seeds (mg kg^{-1})
Eston	50	33.6	2(13)	231.8	33.5	33.2
L01-827	55	22.25	2(18)	12.8	6.4	26.0
LR 59-2	53	23.0	1	10.6	13.6	33.5
LR59-15	56	33.0	3(60)	66.8	18.4	40.9
LR59-27	53	31.2	1	196.3	22.2	32.3
LR 59-42	51	23.4	1	50.7	15.3	31.6
LR 59-55	57	26.4	2	52.4	15.0	37.3
LR 59-60	52	19.8	1	35.4	12.6	38.3
LR59-76	51	25.6	1	70.0	13.9	34.1
LR 59-81	53	26.8	1	53.5	17.2	41.3
LR 59-112	49	31.8	3(115)	131.4	21.0	39.8
LR59-126	54	27.6	2	177.1	21.7	31.3
LR 59-127	50	14.8	1	104.5	21.3	33.6
LR 59-130	53	15.2	1(8)	13.1	9.7	31.3
LR 59-133	54	33.8	2	236.0	22.6	27.0

Note: Nodulation data was taken from single plots; ICARDA nodulation descriptor scale was followed (Nodule number: None =0, Less than 10 = 1, 11-20 = 2, More than 20 = 3)

*Seeded on May 30, 2014

*Because of poor seed germination, 2015 field trial was abandoned.